Physiological performance and adaptive capacity of kelp (Laminariales) in a changing environment

by

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Declarations

Statement of originality

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Statement of co-author contributions

Chapters 2-5 of this thesis has been prepared as peer-reviewed manuscripts. On all accounts, the experimental design and implementation, data analysis and interpretation, and manuscript development was the responsibility of the candidate and carried out in consultation with supervisory team and other third parties. Contributions are detailed below.

Chapter 2: Dr. Jeffrey Wright (primary supervisor) and Prof. Craig Johnson (co-supervisor) contributed conceptual knowledge of macroalgal physiology, experimental design and culturing techniques in addition to multivariate statistical methods. Contributing authors and reviewers provided comments on this manuscript.

Chapter 3: Dr. Jeffrey Wright (primary supervisor) and Prof. Craig Johnson (co-supervisor) contributed conceptual knowledge of macroalgal ecophysiology and culturing techniques. Contributing authors provided comments on this manuscript.

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Chapter 4: Dr. Jeffrey Wright (primary supervisor) and Prof. Craig Johnson (co-supervisor) contributed conceptual knowledge of evolutionary adaptation and heritability of fitness traits, as well as experimental design of breeding experiments and culturing techniques. Contributing authors provided comments on this manuscript.

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Abstract

Kelp ‘forests’ form the foundation of many temperate reef ecosystems. As ecosystem engineers, kelp modify abiotic conditions, create habitat for other species and support highly diverse communities. Climate change represents a threat to kelp ecosystem function, stability and biodiversity and on the east and west coasts of Australia recent warming events have caused significant damage to kelp forests. The south east coast of Australia is warming at approximately four times the global average and the continuation of this warming will likely have further negative impacts on the health and distribution of kelp forests and the ecosystems they create.

Climate change is a multifactor stressor but multifactor studies to determine the impact of climate change on biota are rare. This thesis examines the physiological response and adaptive potential of ecosystem engineering kelps *Macrocystis pyrifera* & *Ecklonia radiata* under relevant multifactor climate change scenarios in south eastern Australia. I assessed a range of physiological parameters to understand the multivariate response in these kelp to higher temperature, reduced nitrates and higher light, incorporating growth rates, photophysiology (as derived from PAM fluorometry and pigment concentrations), nutrient profiles (concentration of C and N, C:N ratios and isotopic signatures of δ13C and δ15N), and nucleic acid levels (RNA and DNA concentrations and RNA:DNA ratios). This approach enabled a holistic evaluation of kelp performance to climate change and also, a test of the applicability of the Growth Rate Hypothesis (GRH) in predicting future kelp distribution. For temperate seaweed, the GRH predicts that selection will favour rapid growth in high latitudes where light is limiting, hence greater nutrient requirements. In this context, it was anticipated that longer periods of warm, oligotrophic East Australian Current (EAC) waters acting simultaneously with a reduction in kelp canopy (increased light) will interact to negatively impact kelp physiological performance, growth and survival.

Chapter 2 examined the effects of temperature, nitrate and light on the growth and physiological performance of *Macrocystis pyrifera* from Tasmania. High temperatures led to down regulation of photosystem II (PSII) as well as photosystem impairment when combined with low light. High light photoinhibition occurred in temperatures above 12 °C. These deleterious effects were characterised by excessive tissue necrosis and mortality. RNA concentrations were associated with stressful conditions but were decoupled from growth, showing no support for the GRH in this species. As expected, optimum growth occurred at

lower temperatures but unexpectedly at low nitrates, perhaps reflecting an adaptive response to the typically low ambient nutrient levels that occur on the east coast of Tasmania.

Chapter 3 investigated the influence of temperature, nitrate and light on the widely distributed kelp *Ecklonia radiata* from two different locations (bioregions). Temperature drove significant variation in PSII metrics overall although optimum PSII performance occurred at low temperatures for Tasmanian *E. radiata* only, whilst light and nitrate had few significant main or interaction effects. Growth was driven by temperature (Tasmanian) or light (NSW). This chapter highlighted the importance of considering latitudinal variation in responses to climate change but also showed a lack of support for the GRH in this species.

Chapter 4 examined the adaptive potential of Tasmanian *Ecklonia radiata* to climate change across haploid and diploid life-cycle stages. There was strong family-level variation in growth, reproduction and photosystem traits in gametophytes and sporophytes, indicating the potential for adaptive responses to climate change. Furthermore, there were significant genotype x environment interactions for some traits indicating families will respond differently to changes in temperature and light. The high adaptive potential suggested by this study could enhance the resilience of *E. radiata* to climate change.

Overall, this thesis indicates that increasing temperatures will continue to impact the performance and distribution of kelp, particularly in Tasmania where there were strong effects of high temperature on both *E. radiata* and *M. pyrifera*. This thesis also highlights the importance of multifactor studies in determining additive effects of climate stressors on kelp and the complex nature of physiological responses to these stressors. Finally, the high family-level variation of fitness traits in the key early life-history stages highlighted adaptive potential in *E. radiata* to climate change.

Chapter 1 | Introduction

Climate and climate variation shapes the worldwide distribution and demography of species (Parmesan *et al.*, 2000; Walther *et al.*, 2002). Ocean temperature is a primary driver determining the distribution of most marine species (Sunday *et al.*, 2012) although other factors such as light and nutrients are also important (Harley *et al.*, 2006). Mounting evidence of distributional shifts to higher latitudes due to climate change has been documented for many terrestrial (Loarie *et al.*, 2009), aquatic (Perry, 2005) and marine species (Poloczanska *et al.*, 2013). Individual organisms can persist in the face of short-term seasonal fluctuations in environmental factors by phenotypic alteration (plasticity, West-Eberhard, 1989). However, under climate change, organisms are likely to be subjected to conditions at the upper limits of their thermal tolerance, and because plasticity can represent a trade-off with other important biological processes, population resilience can be eroded (Steneck *et al.*, 2002; Wernberg *et al.*, 2010). Negative responses of foundation species (i.e. ecosystem engineers) to climate change has major consequences for associated ecological communities due to altered provision of resources, reduced ecological function and, in extreme cases, total ecosystem shift or loss (e.g. phase shift, Folke *et al.*, 2004). Conversely, populations can show adaptive potential under selective pressures through phenotypic or molecular (evolutionary) responses Most organisms alter phenotype to maintain optimum function within a range of abiotic conditions, outside of which may cause impaired function or mortality, while some species are capable of maintaining function for limited periods under severe stress. Over generations, evolutionary responses to stress are underpinned by heritability of important fitness traits (Hartman *et al.*, 2001; Via & Lande, 1985; Hoffmann & Merilä, 1999) Ultimately, the phenotypic and evolutionary response of species will determine

their demography and distribution, and thus these factors are important to understand in the context of future climate change.

Detailed physiological studies can determine a species' performance and tolerance limits to changes in abiotic factors and may be used to extrapolate population-level responses to predicted climate change (Pearson & Dawson, 2003). Controlled laboratory studies can resolve the impacts of climate change on sub-organismal traits of marine macroalgae such as relative growth rates, photosynthetic characteristics of photosystem II (PSII), chlorophyll concentrations, carbon and nitrogen related parameters, nucleic acid concentrations, reproduction and development traits. In seaweeds, these traits all exhibit some degree of plasticity (Flukes *et al.*, 2015). Moreover, examining responses across multiple traits gives a clearer indication of 'whole organism' response (Forsman, 2015).

Until recently, most climate change – physiology studies examined the effect of single factors (e.g. temperature, UV, or acidification) on the physiology of species from single populations to indicate climate impacts across a species distribution (e.g. climate envelope models: Hijmans & Graham, 2006). However, since climate change is inherently a multifactor phenomenon and the potential for additive, synergistic and/or antagonistic effects of multiple stresses is becoming more widely recognised, multifactor studies enable more realistic predictions of species performance under climate change (see review Przeslawski *et al.*, 2015). Furthermore, given biogeographical performance of a species differs across geographic boundaries and temperature zones (Clements & Shelford, 1939; Hutchins, 1947; Breeman, 1988), examining performance of individuals from only a single population may not provide useful information on responses of individuals from other populations as widely distributed species often exhibit a high degree of variation in physiology, phenology and life-history traits across their range (e.g. Zhang & Marshall, 1994; Li *et al.*, 1998; Pilon *et al.*, 2003).

Predicting physiology across latitudes: the Growth Rate Hypothesis (GRH)

The physiological performance of organisms is dependent on the allocation of nutrients and the ability of individuals to maintain stoichiometrically balanced C:N:P ratios (Sterner & Elser, 2002). Species that inhabit a wide latitudinal range exhibit variable C:N:P ratios and thus have varying N and P requirements. The GRH posits that at higher latitudes autotrophic organisms with higher growth rates will be selected for as a means to compensate for a shorter growing season, thus they will be more susceptible to N and P limitation and increased demand on resources for rapid growth (Kerkhoff *et al.*, 2005; Lovelock *et al.*, 2007). As P-rich ribosomal RNA is a basic requirement for protein synthesis in growth and DNA cell concentrations are presumed to be constant (Dortch *et al.*, 1983), RNA:DNA ratios are used as a proxy for growth potential. Application of this theory has had limited examination in marine species, but so far, is supported in invertebrates and microalgae (Dortch *et al.*, 1983; Lepp & Schmidt, 1998; Elser *et al.*, 2000a; Lovelock *et al.*, 2007), and rapidly-growing seaweed (Giordano *et al.*, 2015). The limited testing to date of this hypothesis for large, slow growing seaweeds does not provide any support for the GRH (Reef *et al.*, 2012; Flukes *et al.*, 2015), but it is yet to be tested for laminarians (kelp). It is useful to test the GRH in kelp to determine whether these important organisms are likely to be susceptible to nutrient limitation under climate change and whether the GRH is a good model to describe latitudinal variation in kelp ecophysiology.

Adaptation to climate change

Organisms respond to environmental stress by phenotypic alteration (short-term response) or evolutionary adaptation (long-term response). Phenotypic responses to environmental stressors include alterations in phenological, behavioural, physiological and morphological traits (e.g. Nylin & Gotthard, 1998; Bradley *et al.*, 1999; Hoffmann & Merilä, 1999; Menzel & Fabian, 1999; Pulido *et al.*, 2001; Li & Denny, 2004; McGaugh *et al.*, 2010) to enable

optimum function in heterogeneous or novel environments. For example, the upper temperature thresholds for a species may be well above that which is experienced naturally, and phenotypic responses allow for persistence under short-term extreme conditions. Whilst phenotypic mechanisms exist to deal with acute stress events, it may be too energetically expensive to mount these responses to chronic stress events such as long-term heatwaves, eventually leading to reduced resilience of populations to other stressors and phase shifts to undesirable ecological states (Ling *et al.*, 2009; Vergés *et al.*, 2016).

In addition to short-term plastic response, long-term population-level resilience to novel selection regimes is dependent on evolutionary adaptation (Hoffmann & Sgro, 2011; Kelly & Hofmann, 2013; Reusch, 2014). Many studies of climate-change impacts focus on the change in performance of quantifiable traits to elucidate the extent of phenotypic plasticity, however relatively few consider the adaptive potential of species (Munday *et al.*, 2013) which includes 1) quantitative genetic variation in functional traits; and 2) differential performance of genotypes across environments (Bowman, 1972). For example, terrestrial plants show quantitative genetic variation (i.e. heritability) in ecologically important traits such as leaf thickness and development rates (Jump & Penuelas, 2005; Agrawal *et al.*, 2008), whilst genotypic-mediated responses in quantitative traits relating to phenology, morphology and physiology to environmental stress have been observed in marine invertebrates (Galletly *et al.*, 2007; Pease *et al.*, 2010; McKenzie *et al.*, 2012).

The scenario

Southeast Australia harbours seaweed beds of the Great Southern Reef which are dominated by kelp (laminarian) forests. The two kelp species found in this region have distinct biogeographic distributions. Rear-edge populations of *Ecklonia radiata* and *Macrocystis pyrifera* occur in Queensland and southern Victoria respectively and their distribution extends to the continental limits of southern Tasmania. The region is renowned as a ‘hotspot’

for both marine biodiversity and ocean warming, and until recently has been characterised by cool nutrient rich Southern Ocean waters during winter and early summer with the possibility of seasonal incursions of warm and nutrient-poor East Australian Current (EAC) water. However the region is becoming increasingly dominated by EAC water, and for this reason has been warming rapidly since 1950 (Hobday & Pecl, 2014) and is predicted to continue to warm at almost four times the global average (Ridgway, 2007a) due to shifting wind stress in the Southern Ocean affecting the South Pacific gyre and an altered dynamic of the EAC separation point off the central NSW coast resulting in larger and/or more frequent eddies of EAC water propagating southwards (Cai *et al.*, 2005; Oliver *et al.*, 2014). The warm waters of the EAC are oligotrophic, with nitrate (NO_3^-) levels typically <0.5µm and often undetectable (Harris *et al.*, 1987). As the impacts of climate change will be most severe in places where biodiversity and warming hotspots coincide (Wernberg *et al.*, 2011a, 2012a), and given the recent decline of up to 95% of the giant kelp *Macrocystis pyrifera* in this region (Johnson *et al.*, 2011), it is crucial to understand how local kelps will respond in the future to climate change in order to elucidate the wider impacts of predicted climate shifts on temperate marine ecosystems and the communities they support. This understanding must extend across seaweed distributions as biogeography plays a critical role in how seaweeds respond to stress (van den Hoek, 1982, 1984; Breeman, 1988; Lüning *et al.*, 1990; van den Hoek & Breeman, 1990; van den Hoek *et al.*, 1990). Furthermore, as kelps engineer their own light environment (Wernberg *et al.*, 2011a), any change to kelp bed density will likely impact the understory light environment where recruitment occurs. Thus understanding how the consequences of climate change (increased temperature, reduced nitrate levels, and increased light) will affect the survivorship and growth of juvenile kelps, including their adaptive potential, is of crucial importance to managing temperate marine ecosystems in south east Australia.

Thesis structure

This thesis examines the potential impacts of climate change on the physiology and adaptive capacity of kelp (Order: Laminariales), using two important kelp species from southeast Australia, *Macrocystis pyrifera* and *Ecklonia radiata*, and assesses the adaptive potential of Tasmanian *E. radiata*. The work examines a range of physiological metrics and assesses whether physiological patterns are consistent with the Growth Rate Hypothesis (GRH).

It is important to note that each chapter of this thesis is prepared in a format for stand-alone publication, hence repetition of detailed methodological and other information is unavoidable, particularly across chapters 2 & 3.

Chapter 2 examines the interaction among three abiotic factors (temperature, nitrate, and light) on the univariate and multivariate physiological plasticity of Tasmanian *Macrocystis pyrifera* in a controlled laboratory experiment. This chapter discusses the implications of the results in the context of *in-situ* performance under environmental conditions associated with projected climate change.

Chapter 3 examines the interactive effects of the same three abiotic factors on multivariate ecophysiological traits of two distinct populations of *Ecklonia radiata* from two latitudes (NSW and Tasmania). This chapter also explores whether the two populations exhibit differential physiology under climate change as would be predicted by the GRH.

The final experimental chapter (Chapter 4) investigates the adaptive capacity of *E. radiata* in its microscopic stages. Family-level (genotypic) variation of important quantitative traits are examined in addition to a 'family x environment' experiment to test for genotypically-mediated responses to different environmental combinations of temperature and light. The results are discussed in the context of adaptive potential under environmental stress.

The final chapter explores the current limitations for researching ecophysiology of kelps to determine climate change impacts by synthesising the results within the broader context of seaweed research. The combined results of the thesis work lends support for multifactor, multivariate approaches to seaweed physiological research, whilst integrating lab and field experiments to provide a better predictive ability of seaweed ecophysiology under climate change and to bolster knowledge of seaweed biology and biosynthetic dynamics. The work highlights the need for greater focus on assessing the adaptive capacity to abiotic stress across populations, seasons, and life-stages.

Chapter 2 | Physiological response to temperature, light and nitrates in the giant kelp *Macrocystis pyrifera*, from Tasmania, Australia

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2.1 Abstract

Climate change is characterised by multiple abiotic forcings acting simultaneously on biotic systems. In marine systems, temperature appears to drive much of the observed change in biotic communities subject to climate change, but this may reflect the focus of most studies only on temperature without consideration of other environmental variables affected by climate change. The giant kelp (*Macrocystis pyrifera*) was once abundant in eastern Tasmania, forming extensive habitat of ecological and economic importance but recent extensive population decline has occurred. Southerly incursion of warm oligotrophic East Australian Current (EAC) water has increased in frequency and intensity into this region which has warmed at ~4-times the global average, and the warming trend is predicted to continue. This study investigated the single and combined effects of temperature, light, and nitrate availability on the physiology of *M. pyrifera* sporelings in a laboratory experiment. Determination of relative growth rate, photosystem II characteristics, pigments, elemental chemistry, and nucleic acid characteristics over 28 days showed that all experimental factors affected sporeling physiology. Temperature and light drove much of the observed variation related to performance characteristics, and rapid deterioration of kelp tissue was a consequence of temperature stress (high temperature), photoinhibition (high light), and low-light, accompanied by impaired photosynthetic efficiency and increased RNA concentration,

presumably associated with production of photoprotective proteins. Surprisingly, higher relative growth rates were observed in low nitrate treatments. These findings suggest that increasing temperature will have ongoing negative effects on remaining *M. pyrifera* populations in Tasmania leading to further population decline but these effects will be mediated by local variation in light and nutrient conditions.

2.2 Introduction

Climate change is transforming marine ecosystems, causing shifts in species ranges (Burrows *et al.*, 2011), declines in biodiversity (Sala *et al.*, 2000) and changes to ecosystem structure and functioning (Wernberg *et al.*, 2011b, 2012a; Doney *et al.*, 2012). Negative impacts of climate change on marine foundation species (Dayton, 1972) or 'ecosystem engineers' (see Jones et al. 1994) such as corals, seagrasses, mangroves and seaweeds, are likely to be particularly important as these species form the basis of hierarchically organised species-rich communities (Crain & Bertness, 2006; Schiel & Foster, 2015) that support extensive economic and social wellbeing (Ewel *et al.*, 1998; Cesar, 2002; Bennett *et al.*, 2016; Dewsbury *et al.*, 2016). Although temperature appears to be a major factor impacting the performance and physiology of marine organisms, climate change encompasses shifts in other environmental factors (i.e. light and nutrient supply) important for biological functioning (e.g. biosynthesis and metabolism), and which may act on their own or in combination with temperature (Poloczanska *et al.*, 2007). Multiple forcing factors complicate the ability to predict, test and interpret the impacts of climate change, creating uncertainty for adaptive management strategies (Straub *et al.*, 2016).

Habitat-forming seaweeds are a dominant feature of temperate marine ecosystems and form the basis of productive and diverse communities (Mann, 1973; Schiel & Foster, 1986; Bruno & Bertness, 2001; Steneck *et al.*, 2002; Bruno *et al.*, 2003). The south east region of Australia is an ocean warming 'hotspot' (Hobday & Pecl, 2014) and encompasses a substantial portion

of the Great Southern Reef, one of the most productive temperate reef zones in the world (Bennett *et al.*, 2016). Historically, this region has been warming rapidly since 1950 (Hobday & Pecl, 2014) and is predicted to continue to warm at almost four times the global average (Ridgway, 2007a) due to shifting wind patterns and ocean currents which cause more frequent and intense southerly incursions of East Australian Current (EAC) (Cai *et al.*, 2005; Oliver *et al.*, 2014). The warm waters of the EAC are oligotrophic, with nitrate (NO_3^-) levels typically <0.5µm and often undetectable (Harris *et al.*, 1987). As seaweeds rely on seasonal nutrient loading for growth and other metabolic processes (Chapman & Craigie, 1977; Gerard, 1982; Wheeler & Srivastava, 1984) increased exposure to EAC conditions are likely to exert both temperature and nutrient stress on canopy-forming kelps.

Macrocystis pyrifera (C. Agardh) is the world's largest and fastest growing seaweed. It is found from the low intertidal zone to around 30 metres depth in all continents in the southern hemisphere (except Antarctica) and the west coast of Northern America (Graham *et al.*, 2007), where it forms giant kelp 'forests' in cool-temperate waters. Its large-scale distribution is determined by temperatures between 4 – 20 °C and nitrate concentrations > 1 µM NO_3^- (Schiel & Foster, 2015), while local-scale processes such as grazing, storms and upwelling events play major roles in driving kelp forest dynamics (Dayton *et al.*, 1984, 1998, 1999; Ebeling *et al.*, 1985; Steneck *et al.*, 2002; Krumhansl *et al.*, 2016). In Australia, *M. pyrifera* is confined to the south eastern part of the continent with the largest population occurring in Tasmania where it was once a prominent habitat type in eastern Tasmania at depths of 8 – 22 metres (Edgar, 1984). However forests forming dense surface canopies have declined by up to 95% in the past 60 years (Johnson et al. 2011) associated with the increased influence of warm oligotrophic EAC waters in the region. In response to the decline, *M. pyrifera* was listed as an endangered marine habitat type under the federal Environmental Protection and Biodiversity Conservation Act 2012 (EPBC Act 1999). The need for better predictions of the

future condition of giant kelp forests under climate change is important for management and/or adaptation by humans, and resolving impacts of climate change on the physiological functioning of *M. pyrifera* in eastern Tasmania is one element of this goal.

In general, increasing temperatures lead to reduced fitness in temperate seaweeds (Hatcher *et al.*, 1987; Serisawa *et al.*, 2002; Andersen *et al.*, 2013; Jueterbock *et al.*, 2014) and further temperature driven declines of *M. pyrifera* populations are predicted in south east Australia (Johnson *et al.*, 2011). However, as light and nutrients also play key roles in seaweed physiology these factors must be addressed alongside temperature, and the potential interactions among them considered. Multifactor climate impact studies with seaweeds reveal both synergistic or antagonistic effects between factors (e.g. among temperature, salinity, UV intensity, desiccation, wave action, and light) on the growth and photosynthesis in *Fucus spp.* (see review Wahl et al. 2011), but also show additive effects in some cases (e.g. temperature and UV inhibits *E. radiata* growth and photosynthesis rates: Xiao et al. 2015). Increased influence of EAC waters in Tasmania will likely lead to kelp canopy destabilisation and thinning and thus increased light levels (Wernberg *et al.*, 2010). Adult *M. pyrifera* engineer the understory light environment, and thus for sporelings light can be either limiting under a thick canopy (Dean & Jacobsen, 1984; Kinlan *et al.*, 2003) or high and photo inhibitory under a disturbed or absent canopy (Graham, 1996; Altamirano *et al.*, 2004), and therefore resolving how temperature and light are likely to interact is important. Additionally, little is known of *M. pyrifera* nutrient utilisation dynamics in Tasmania. The Growth Rate Hypothesis (Elser et al. 2000) would posit that seaweeds at higher latitudes will be more susceptible to nutrient limitation because selection favours higher instantaneous growth rates in shorter growing seasons, requiring higher P-rich RNA concentrations and a greater reliance on N for stoichiometry balance to maintain photosynthesis machinery. Assuming DNA tissue concentration is constant, organisms with higher growth potential should have higher RNA:DNA ratios and higher N-limitation thresholds (Dortch *et al.*, 1983; Elser *et al.*,

2003). Thus, it is useful to assess how nutrients and light interact with temperature to affect *M. pyrifera* physiology under climate change scenarios.

This study examines the interaction of temperature, light, and nitrate on the physiology of juvenile *M. pyrifera*. The aims were to test whether: 1) the effects of increased temperature on *M. pyrifera* physiology will be particularly severe in south east Australia due to synergistic effects of reduced nitrates and increased light due to a decline in canopy cover, and 2) whether RNA concentration, and thus RNA:DNA ratios, will correlate with growth rates due to increased protein synthesis.

2.3 Materials & methods

2.3.1 Collection and in situ measurements

M. pyrifera sporelings (50 – 150 mm in length) were collected from *M. pyrifera* beds at Fortescue Bay, Tasmania (43.1230°S, 147.9764°E) in September 2012 at a depth of ~10 m. At the time of collection *in situ* water temperature was ~13°C and photosynthetically active radiation (PAR) under the canopy was approximately 30 µmol photons m^{-2} s^{-1} (Odyssey PAR logger, Dataflow Systems Pty Ltd Christchurch, NZ). Sporelings were transported in seawater-filled coolers to the IMAS laboratory facility and held at a temperature of 13 °C for 24 hours under low light (12:12 light – dark at 10 µmol photons m^{-2} s^{-1}) to slow respiration and photosynthesis rates and reduce the risk of temperature, oxygen, and nutrient stress (Peckol, 1983). During collections, *in situ* baseline physiology was measured from three haphazardly selected sporelings using a PAM fluorometer, and tissue was collected for pigment, chemistry, and nucleic acid analysis in the laboratory (see *2.3.4 Physiological measurements).*

2.3.2 Experimental design and growth conditions

The response of *M. pyrifera* sporelings to temperature, light, and nitrate levels were determined using a three-way factorial design, with main effects of temperature (12, 17, 22

°C), irradiance (6, 30, 80 µmol photons m^{-2} s^{-1}), and nitrate concentration (0.5, 3.0 µM NO_3^-). *M. pyrifera* sporelings were grown for up to 28 days in the experimental treatments in the laboratory which is sufficient time to see changes in seaweed growth and elemental stoichiometry (Flukes *et al.*, 2015), with three independent replicates of each temperature-irradiance-nitrate combination.

Upon arrival at the laboratory, sporelings were divided between three holding tanks containing 0.2 µm filtered seawater, aerated and shaded with flyscreen mesh and assigned one of three temperature controlled rooms. Incremental changes to temperature (approx. 2.0 °C.day^{-1}) were made until experimental target conditions were reached. An earlier pilot trial indicated acute physiological stress in kelp sporelings under sudden increases in temperature and light. Consequently, incremental changes to temperature (2 °C day^{-1}) were made until the three experimental target temperatures were reached. After 3 days acclimation, thalli were haphazardly selected from the tubs and two individuals were placed into each glass beaker (2000 mL) containing growth media and subject to a particular treatment. Thalli were free-floating, as pilot trials showed no difference in physiological performance between vertically oriented and free-floating thalli. The media was aerated to ensure sufficient mixing and disturbance of the diffusion boundary layer around thalli. Beakers were shaded by multiple layers of flyscreen to achieve irradiance of 10 µmol photons m^{-2} s^{-1}. Irradiance was increased for high light treatments by removal of layers of flyscreen over an additional two days by 10 µmol photons m^{-2} s^{-1}.day^{-1} until the target of 30 µmol photons m^{-2} s^{-1} was reached, and similarly more flyscreen was added to achieve the low light treatment of 6.0 µmol photons m^{-2} s^{-1}. Media was replenished every 2-3 days for the duration of the experiment to ensure nutrient levels were maintained.

Growth media (Wright's Chu #10 (WC)) comprised autoclaved, nutrient depleted, 0.2 µm filtered seawater with added base stocks of vitamins, trace metals, potassium hydrogen

sulphate (K_2HPO_4) and sodium nitrate ($NaNO_3^-$) (Guillard & Lorenzen 1972, reviewed by Andersen 2005). Nitrate levels (3.0 µM & 0.5 µM NO_3^-) were chosen based on normal (high) and EAC influenced (low) ranges observed at Maria Island, Tasmania (Rochford, 1984). Manipulation was achieved by modifying the volume of $NaNO_3^-$ added to WC media to achieve target concentrations, whilst keeping the ratio of phosphorus to nitrate stoichiometrically balanced at 20:1 by also manipulating the addition of K_2HPO_4 (Guillard & Lorenzen, 1972), in order to avoid limiting of macronutrients. Aeration of the media in each beaker was used to promote circulation of media around the thalli, with air passing through a 0.2 µm syringe filter to minimise media contamination risk. Temperature-controlled rooms were set to 12, 17 and 22 °C. This range of temperatures was approximated to reflect current temperatures experienced by *Macrocystis* in Tasmania (~12 °C winter & ~18+ °C summer) and projected summer maxima (22 °C) under the highest possible Representative Concentration of Pathways of greenhouse gas emissions (RCP8.5) scenarios at current CO_2 loadings (CSIRO and Bureau of Meteorology, 2015). Light was provided by cool white fluorescent tubes (Sylvania 36W/w41, Danvers, MA, USA) in a 12:12 light:dark (L:D) cycle with an incident irradiance of 6, 30 and 80 µmol photons m^{-2} s^{-1}, simulating light levels in the field under dense, partial, and absent kelp canopy (Flukes et al. *unpub. data*; Tatsumi & Wright, 2016; Fain and Murray 1982; Dayton et al. 1999; Clark et al. 2004).

2.3.3 Physiological measurements

Physiological responses requiring tissue sacrifice were measured from one of the pair of thalli in each beaker at T=0 days (T_0: post-acclimation) and at the end of experiment (T_{end}: up to 28 days after T_0). On both occasions PAM fluorometry measurements were taken, and then tissue was sacrificed for chlorophyll, tissue chemistry, and nucleic acid analysis. At T_0 the second thallus was weighed to enable determination of relative growth when the same individual was weighed at the end of the experiment. Once PAM fluorometry was conducted,

thalli were cut into sections of 10-20 mm^2 for nucleic acid analysis and stoichiometric analysis, while larger sections (20 - 40 mm^2) were required for pigment analysis (see below). All sections were handled with gloves, tweezers, and a scalpel after being washed in dH_2O, then patted dry and prepared for storage before processing. Tissue chemistry sections were immediately placed into a -20°C freezer for further analysis and pigment sections were immediately frozen in darkness at -20°. Nucleic acid sections were placed in RNAlater (Ambion. Inc., Austin, Texas), a non-toxic stabilisation buffer that preserves total tissue RNA and DNA then frozen at -20°C.

2.3.4 PAM fluorometry

Relative photosynthetic performance was estimated from rapid light curves (RLCs) obtained by measuring variable chlorophyll *a* fluorescence in photosystem II (PSII) as a function of photosynthetic active radiation (PAR) using a blue-light diving PAM fluorometer (Walz, Germany). A 'leaf clip' with closable window was attached to thalli just above the meristematic region to maintain uniform spacing between the fibre optic light source and thallus tissue, and to eliminate ambient light interference to ensure consistency of fluorescence measurements. RLCs were generated by an internal PAM software routine where actinic light intensity increased in eight steps of 10 seconds each, measuring effective quantum yield of PSII (ϕ_{PSII}) as a function of PAR at each step. Relative electron transport rate (rETR) was determined by multiplying ϕ_{PSII} by the respective PAR (Schreiber *et al.*, 1994), which estimates the rate of electrons pumped through the photosynthetic chain (Beer *et al.*, 2001). Estimates of electron transport dynamics were determined by plotting rETR against the respective PAR and fitted to a double exponential decay model (see below).

RLCs were determined twice on single thalli under pre-treatments of ambient light and dark-acclimation (approximately 15 minutes or more). Dark-acclimation duration was determined by pilot studies to be approximately 15 minutes, after which more time under dark conditions

showed no further increases in F_m or F_v/F_m, indicating re-oxidation of the electron transport chain and relaxation of the photoprotective mechanisms. Ambient light RLCs reflect the immediate light history and can be affected by ambient irradiance conditions (i.e. cloud cover, canopy shading, water turbidity etc.), whereas dark-acclimated RLCs indicate the inherent state of the photosystem (Ralph & Gademann, 2005). When conducting PAM fluorometry with a leaf clip, local areas of tissue are subject to saturating pulses of actinic light, so it was ensured that the two PAM measurements (i.e. light-acclimated and dark-acclimated) came from different tissue, by moving the PAM leaf clip approximately 1-2 cm from the first measurement position between readings.

RLCs can be described and compared by characterising the photosynthetic response (raw rETR data) as a function of light, determined by the initial linear response and the region of photoinhibition (Ralph & Gademann, 2005). RLC parameters were derived by fitting the raw data to Platt et al.'s (1980) double exponential decay function to calculate maximum electron transport rate ($rETR_{max}$) and saturating light intensity(E_k) using the following equation:

$$P = P_S\left(1 - exp\left(\frac{-\alpha E_d}{P_S}\right)\right) \times exp\left(\frac{-\beta E_d}{P_S}\right)$$

where P is the photosynthetic rate (rETR), α the initial slope before the onset of saturation, E_d is the incident downwelling irradiance of the PAM internal halogen light, β characterises the slope region where PSII declines after photoinhibition (Henley, 1993), and P_s is a scaling factor defined as the maximum potential rETR. Parameters $rETR_{max}$ (maximum electron transport rate) and E_k were estimated as per the Platt et al. (1980) equation using a nonlinear least-squares function in the 'R' software environment (v 3.0.0) to fit the models. To ensure convergence the regression model settings were as follows: iterations = 100; stepsize = 1/1024; tolerance = 0.00001; initial seed value for P = maximum rETR derived from raw

data, α=slope of linear regression fitted to first three points of raw data (typically in the range 0.7-1.0).

The maximum quantum yield (F_v/F_m) was determined from dark-acclimated measurements where the minimum (F_0) and maximum (F_m) fluorescence were used to calculate variable fluorescence (F_v), then determined the intrinsic potential quantum efficiency of PSII (F_v/F_m).

2.3.5 Pigments

Frozen samples were thawed, patted dry, and 100-150 mg of tissue was weighed (to the nearest 0.01 mg) and placed into a 15 ml vial containing 5 ml of N,N-Dimethylformamide (DMF: Sigma Aldrich Pty Ltd, Castle Hill, Australia) to facilitate pigment extraction. Extraction vials were pre-wrapped in aluminium foil and samples were processed rapidly in a fume hood under low light to avoid pigment damage from ambient light then placed in the freezer under total darkness at -20 °C for 96 hours for pigment extraction to occur.

2.3.5.1 Chlorophyll a and c

A 3 ml aliquot of pigment extract was pipetted into a centrifuge tube and centrifuged for 8 minutes at 8,000 rpm. Chlorophyll *a* and *c* content of the supernatant was determined spectrophotometrically (wavelengths: 664.5, 631 and 582 nm) using a Dynamica HALO RB-10 Spectrophotometer and processed using UV Detective software (v1.1). Blanks of 100% DMF were read every ten readings. In accordance with manufacturer's instructions, where absorbance read above 1.000, supernatant was diluted with DMF until absorbance dropped below 1.000. Dilution factor was recorded and factored into the final calculations for pigment concentration. Chlorophyll *a* and *c* concentrations were calculated using the recommended absorption coefficients following Inskeep and Bloom (1985) and Seely et al. (1972).

2.3.5.2 Fucoxanthin

Fucoxanthin content was determined from the remaining ~2 ml extractant. Aliquots of 2 µl of extract were injected using Waters Acquity H-series Ultra High Performance Liquid

Chromotography (UPLC, Waters, Milford MA, USA) coupled to a Waters Acquity Photodiode Array detector (PDA) with a Waters Acquity UPLC Ethylene Bridged Hybrid (BEH) C18 column (2.1 mm x 100 mm x 1.7 micron particles). Mobile phases comprised a gradient mixture of three solvents prepared by Merck Chemicals (Merck KGaA, Darmstadt, Germany), viz. acetic acid (1%), acetonitrile, and 80:20 methanol:hexane (Merck KGaA, Darmstadt, Germany). Initial conditions were held for 3.5 minutes in a 20% acetic acid solvent and 80% acetonitrile solvent, followed immediately by 80% acetonitrile and 20% methanol:hexane solvent which was then held for a further 5.5 minutes, followed by 3 minutes re-equilibration to original conditions. The column was held at 35° C and the flow rate was 0.35 ml min^{-1}. The PDA was monitored continuously over the range 230 to 500 nm. Under these conditions fucoxanthin eluted at 2.3 minutes. Initial calibration of the visible ultra-violet (UV-Vis) response at 440 nm for fucoxanthin was carried out on a freshly prepared standard solution (Sigma Aldrich, Castle Hill, Australia) made up at 1.26 μg ml^{-1} in methanol. Chromatograms were extracted at 440 nm from the raw data using Waters TargetLynx V4.1 software, and the area of the fucoxanthin peak was recorded and converted to mg ml^{-1} fucoxanthin before conversion to mg g^{-1} wet weight.

2.3.6 Elemental stoichiometry (N, C)

Frozen samples were thawed, patted dry, and then freeze dried with weighing every 12-24 hours (to 0.01 mg) until deemed anhydrous when no further weight loss was detected. Samples were ground and homogenised in a mortar and pestle, and approximately 5 mg of powder was placed into tin cups, which were folded gently prior to analysis. Carbon, nitrogen and isotope signatures ($\delta^{13}C$ and $\delta^{15}N$) were measured using Thermo Gas Chromatograph coupled to a Finnigan Mat Delta S isotope radio mass spectrometer in continuous flow mode at CSIRO, Hobart. Results were calculated as follows and are presented in standard sigma notation:

$\delta^{13}C$ or $\delta^{15}N$ (‰) = $\left[\frac{R_{sample}}{R_{standard}} - 1\right]$ where R = $\frac{^{13}C}{^{12}C}$ or $\frac{^{15}N}{^{14}N}$

Standards were replaced and run every 12 cycles with Pee Dee Belemnite (PDB) used as a standard for carbon and air as a standard for Nitrogen.

2.3.7 Nucleic acids

Absolute RNA and DNA was determined to obtain RNA:DNA ratios as a proxy for "growth potential" by extraction of total nucleic acids and RNA/DNA components from a single tissue sample. A piece of algal tissue preserved in RNAlater weighing between 1-5 mg (w/w) was patted dry, weighed and homogenised in a round-bottom 2 µl centrifuge tube (Eppendorf Safe-lock microcentrifuge tube) using a drill pestle in an extraction buffer comprising 500 µl urea (4M), 1% sodium dodecyl sulphate (SDS), trisodium citrate (1 mM), sodium chloride (0.2 M), and 5 µl of proteinase K (Urea/SDS buffer). To ensure stabilisation of nucleic acid, digestion of RNAses by Proteinase K and complete cell lysis, the homogenised solution was held at 37 °C for 10 minutes then placed immediately onto ice. Impurities (e.g. chlorophyll, phenolic compounds, salts, detergents in urea/SDS) were removed by vortexing the solution with 750 µl of ammonium acetate, followed by centrifugation of 5 minutes @ 14,000 RCF. The resulting supernatant was decanted into a 1.5 ml tube to which 700 µl isopropanol was added and the tube was gently inverted 40 times to aid complete mixing of total nucleic acids (tNA) and isopropanol. The total tNA was pelletised by centrifuge (10 mins @ 14,100 RCF) and the pellet was washed twice in a 75% ethanol (EtOH) solution, then resuspended in 200 µl molecular grade H_2O at 55°C for 10 minutes, and separated into two 100 µl aliquots.

To isolate RNA, a solution of 80 µl molecular grade water, 5 µl DNAse (DNase I – Biolabs M0303L) with 20 µl buffer (New England Biolabs - B0303S) was added to one aliquot for total DNA digestion, whilst 100 µl water and 5 µl RNAse (Sigma Aldrich - R6148-25ML) was added to the second aliquot to digest RNA for total DNA isolation. To facilitate

digestion, aliquots were incubated at 37 °C for 20 minutes then stabilised on ice. Isolated nucleic acids were stabilised and extracted by vortexing (10 s) with 400 µl of urea/SDS buffer, vortexing (15 s) and centrifuging (10 min @ 14,000 RCF) with 200µl ammonium acetate (7.5M), decanting supernatant into 1.5 ml tubes, and binding and pelletising with isopropanol as described in the previous step. RNA and DNA pellets were washed twice in 75% EtOH and resuspended into 100 µL of molecular grade water (RNA) and EB buffer (DNA). RNA and DNA concentrations were measured by fluorescence assays using a Quibit assay probe and fluorometer, and expressed as total RNA and total DNA ($ug.g^{-1}$ wet weight tissue). These values were used to calculate the RNA:DNA ratios.

2.3.8 Growth

Absolute growth and relative growth rates were determined from wet weight measures after first drying the specimen on absorbent paper towel before placing on the scale. Daily relative growth rate was calculated as $R = \frac{\log_{e\,2} W - \log_{e\,1} W}{{}_2T - {}_1T}$ (Evans, 1972), where W is weight in grams and T is time in days.

2.3.9 Statistical analyses

Tissue necrosis during the experiment led to poor tissue condition in some sporelings which resulted in unrealistic physiological measurements (PSII, chemistry and nucleic acid) and a number of individuals, particularly from high temperature and high light treatments, were not able to be measured for certain metrics. Therefore the planned fully factorial univariate analyses testing for temperature x light x nitrate effects after 28 days of experimental treatments were not possible for some metrics. Data were partitioned into three different analyses:

1) at low temperature (12 °C), two-way ANOVAs determined the effects of light (fixed: 6, 30 and 80 µmol photons m^{-2} s^{-1}) and nitrate (fixed: 0.5 and 3.0 µM NO_3^-) at T_0 and T_{end} on PSII, pigments, elemental chemistry, nucleic acids, and relative growth rates;

2) at T_0, three-way ANOVAs tested the effects of temperature (fixed: 12, 17 and 22 °C), light (fixed: 6 and 30 µmol photons m^{-2} s^{-1}, i.e. 80 µmol photons m^{-2} s^{-1} was not included) and nitrate (0.5 and 3.0 µM NO_3^-). The same analyses were done at T_{end} except for treatments subject to high water temperature (22 °C) which was dropped for all response variables except for pigments for which sufficient material was available;

3) at T_{end}, moderate light (30 µmol photons m^{-2} s^{-1}) occurred across all levels of temperature and nitrate, and thus a two-way ANOVA was conducted across temperature and nitrates for all metrics except pigments, which were incorporated into the second set of analyses.

Assumptions of ANOVA were checked and transformations determined using the Box-Cox method (Box & Cox, 1964). Tukey's HSD post-hoc tests were conducted where there were significant overall results to determine the source of differences between treatment groups.

The multivariate physiological response to treatments was analysed using permutational multivariate ANOVA (PERMANOVA; Anderson 2001) at both T_0 and T_{end} to determine overall treatment effects on joint distributions of response variables. In five instances, determination of $rETR_{max}$ and E_k was not possible due to degraded tissue and so these variables were not included in the multivariate analyses. The design was unbalanced since one replicate could not be included because of missing nucleic acid data. PERMANOVA was conducted on Gower similarity matrices (Gower, 1971) generated from raw data, with 9999 permutations to calculate pseudo *F*-statistics. Terms with negative estimates of components of variation were pooled (Anderson *et al.*, 2008).

2.4 Results

2.4.1 Baseline field measurements

Baseline physiology of *M. pyrifera* of four randomly selected individuals from Fortescue Bay, Tasmania is presented in each figure and represents mean values for each physiological metric *in situ* at time of collection.

2.4.2 Short-term response to acclimation: low temperature treatment (12 °C)

Following short-term acclimation (i.e. at T_0) at 12 °C, two-way ANOVA revealed no effects of nitrate or light separately, nor any light x nitrate interaction, for F_v/F_m, pigments, nitrogen concentration or nucleic acids (Table 2.1A, Figs 2.1 – 2.4). $rETR_{max}$ and E_k were significantly higher at high light compared to low light (80 > 6 µmol photons m^{-2} s^{-1}, Fig. 2.1). Sporelings in moderate light (30 µmol photons m^{-2} s^{-1}) treatments had significantly higher carbon concentration (compared to other light treatments) and C:N ratio (compared to high light only; Table 2.1A, Fig. 2.3). In low light treatments stable isotope ratios of sporeling tissue were less negative for $\delta^{13}C$ (compared to moderate light) and $\delta^{15}N$ (compared to high light; Table 2.1A, Fig. 2.3).

2.4.3 Short-term response to acclimation: multiple temperature treatments

Three-way ANOVAs (excluding the high light treatment) showed no treatment effects at T_0 for chlorophyll *a*, fucoxanthin, and $\delta^{15}N$ (Table 2.2A, Fig. 2.2 – 2.3). $rETR_{max}$ and E_k were significantly higher at moderate light (vs. low) and 17 °C (vs. 12: Table 2.2A, Fig. 2.1). At 22 °C, there were no further increases in PSII traits, but significantly lower F_v/F_m under low light (Table 2.2A, Fig. 2.1), indicating a reduction in PSII integrity at high temperature and low light (temperature x light interaction). Under low light, chlorophyll *c* was significantly higher (Table 2.2A, Fig. 2.2) while carbon concentration and C:N ratios were lower, associated with a less-negative carbon isotope signature (Table 2.2A, Fig. 2.3). At high temperature (22 °C) sporelings subject to low light treatments had higher nitrogen

concentrations (Table 2.2A, Fig. 2.3) but were lower in RNA content (Table 2.2A, Fig. 2.4) than when grown under moderate light. RNA concentrations were higher at 12 °C (vs. 22, but only at low light; temperature x light interaction), and 0.5 µM NO_3^- (vs. 3.0, but only in moderate light; nitrate x light interaction: Table 2.2A, Fig 2.4). There were significant main effects affecting absolute DNA concentration (light: 30 > 6 µmol photons m^{-2} s^{-1}) and RNA:DNA ratios (light and temperature: 6 > 30 µmol photons m^{-2} s^{-1}, and 22 > 17 °C; Table 2.2A, Fig. 2.4).

2.4.4 Longer-term responses: low temperature treatment (12 °C)

2.4.4.1 Photosystem II and pigments

At the end of the experiment (i.e. T_{end}), sporelings grown at low temperature (12 °C) revealed no treatment effects for F_v/F_m or fucoxanthin (Table 2.1B, Fig. 2.1 – 2.2). $rETR_{max}$ was significantly highest under high light – high nitrate combination (light x nitrate interaction), while E_k was significantly higher when sporelings were grown under high light than at moderate light levels (Table 2.1B, Fig. 2.1). Pigment concentrations were higher under low nitrate treatments (chlorophyll *a* only) and low light treatments when compared to moderate light (chlorophyll *c* only: Table 2.1B, Fig. 2.2).

2.4.4.2 Growth, nucleic acids and tissue chemistry

At T_{end} and 12 °C, no treatment effects were found for $\delta^{15}C$, RNA concentration, or RNA:DNA ratios (Table 2.1B, Fig. 2.3 – 2.4). Across all light treatments, high light yielded more negative $\delta^{15}N$ signatures and higher concentrations of DNA (Table 2.1B, Fig. 2.3 – 2.4) compared to other light treatments. Surprisingly, relative growth was significantly higher under the lowest nitrate treatment (Table 2.1B, Fig. 2.4).

2.4.5 Longer-term responses: multiple temperature treatments

2.4.5.1 Photosystem II and pigments

At the end of the experiment, three-way ANOVA showed no treatment effects for F_v/F_m (Table 2.2B, Fig. 2.1). Temperature effects on $rETR_{max}$ (17 > 12 °C) were restricted to moderate light levels only (temperature x light interaction), whilst the effect of temperature on E_k was not dependent on the light level (Table 2.2B, Fig. 2.1). Low light yielded significantly higher chlorophyll *a* and *c* concentrations, but there was no such change in fucoxanthin levels (Table 2.2B, Fig. 2.2). Higher concentrations of chlorophyll *a* and fucoxanthin at 22 °C (vs. 12 °C) only occurred at high nitrate concentrations (temperature x nitrate interaction). Chlorophyll *c* concentration was higher at 12 °C (vs. 22) when nitrate levels were low, and was higher at 0.5 µM NO_3^- treatments (vs. 3.0) but only at 22 °C (temperature x nitrate interaction: Table 2.2B, Fig. 2.2).

2.4.5.2 Growth, nucleic acids and tissue chemistry

Three-way ANOVA revealed no treatment effects on nucleic acid levels (Table 2.2B, Fig. 2.4). Carbon concentration was significantly higher at 30 µmol photons m^{-2} s^{-1} light treatments (vs. 6), but only at low nitrate availability (Table 2.2B, Fig. 2.3). Nitrate treatments significantly influenced nitrogen concentration (3.0 > 0.5 µM NO_3^-) and C:N ratio (0.5 > 3.0 µM NO_3^-) in low light treatments only (light x nitrate interaction). Additionally, C:N ratio was significantly higher under 30 µmol photons m^{-2} s^{-1} (vs. 6) light treatment but only in high nitrates (light x nitrate interaction: Table 2.2B, Fig. 2.3). Significant temperature and nitrate effects were additive for relative growth rates which were higher at 12 °C and low nitrates (Table 2.2B, Fig. 2.4).

2.4.5.2 Longer-term responses: moderate light treatment

At T_{end}, under moderate light levels (30 µmol photons m^{-2} s^{-1}) two-way ANOVA showed no treatment effects on carbon or nucleic acid concentrations (Table 2.3, Fig. 2.3 – 2.4). $rETR_{max}$

and E_k increased with temperature from 12 to 17 °C but not to 22 °C, and was associated with significantly lower F_v/F_m at 22 °C, suggesting down regulation and compromised integrity of photosystem II (Table 2.3, Fig. 2.1). In low nitrate treatments, there were temperature effects on nitrogen concentration (12 = 17 > 22 °C) and the C:N ratio (22 > 12 = 17 °C: significant temperature x nitrate interaction), while at 22 °C nitrate levels affected the C:N ratio (0.5 > 3.0 µM NO_3^-). $\delta^{15}N$ values were less negative under 22 °C (vs. 12 & 17) and 0.5 µM NO_3^- (vs. 3.0: Table 2.3, Fig. 2.3). Relative growth was significantly lower at 22 °C (vs. 12 & 17: Table 2.3, Fig. 2.4).

2.4.6 Multivariate phenotype response

Three-way PERMANOVA revealed significant variation in the multivariate physiological response of *M. pyrifera* sporelings at both T_0 and T_{end} due to interaction effects of various combinations, although there was no evidence of a three-way interaction (Table 2.4). Short-term (T_0) multivariate response differentiated 12 and 17 °C from 22 °C under low light only (a temperature x light interaction: Pseudo-$F_{1,21}$ = 2.29, P = 0.036). The first two eigenvalue correlations of the canonical analysis of principle coordinates (CAP) were 76 % and 29 % and suggested that the variables contributing most to the variation under different treatments were F_v/F_m, DNA and accessory pigments, which increased in the direction of benign environments whereas RNA and elemental chemistry increased in the direction of stressful environments (Fig. 2.5). By the end of the experiment (T_{end}), the multivariate response indicated significant two-way interactions involving all three factors (temperature x nitrate: Pseudo-$F_{2,21}$ = 2.89, P = 0.004; temperature x light: Pseudo-$F_{1,21}$ = 3.14, P = 0.005; and nitrate x light: Pseudo-$F_{2,21}$ = 2.36, P = 0.007). The size of the first four eigenvalue correlations of the CAP analysis were 96 %, 89 %, 80%, and 77 % and suggested that the variables contributing most to the variation under different treatments were relative growth

rate and F_v/F_m and carbon concentration, which increased in favourable environments whilst RNA, pigments and DNA increased under stressful environments.

Table 2.1 – F-test statistics for two-way factorial ANOVA testing for effects of light (3 levels: 6, 30 & 80 µmol m^{-2} s^{-1}) and NO_3^- (2 levels: 0.5 & 3.0 µM L^{-1}) at 12 °C on Tasmanian-sourced juvenile Macrocystis pyrifera photosystem II (PSII), pigments, elemental chemistry and nucleic acids at T_0 A. and T_{end} B. Results of Tukey's HSD tests for significant results are given below the table. Only significant differences are shown (i.e. levels not included in table are not significantly different to all other levels). Abbreviations given in the Tukey test are: high light (HL); moderate light (ML); low light (LL); low nitrate (LN); high nitrate (HN). Degrees of freedom (*df*) for treatment effects (vertical) and denominator (horizontal). Test of significance: *** P < 0.001; ** P < 0.01; * P < 0.05.

A

		PSII			Pigments			Elemental chemistry					Nucleic acid		
Factor		$rETR_{max}$	E_k	F_v/F_m	*Chl a*	*Chl c*	Fuco	%C	%N	C:N	$\delta^{13}C$	$\delta^{15}N$	RNA	DNA	RNA:DNA
	df	11	11	12	12	12	12	12	12	12	12	12	12	12	12
Light (L)	2	**4.88***	**7.35****	0.58	0.95	1.70	1.16	**14.1*****	0.64	**4.76***	**5.18****	**6.90***	1.34	1.47	1.19
Nitrate (N)	1	0.19	1.91	0.13	0.63	0.02	0.03	0.43	1.87	0.46	0.26	0.10	0.58	0.03	0.21
L x N	2	0.49	0.32	1.26	0.03	0.23	0.06	0.33	1.14	0.96	0.23	0.80	2.89	0.59	0.12
Tukey's															
Light (L)		HL>LL	HL>LL					ML>LL=HL		ML>HL	LL>ML	LL>HL			

B

		PSII			Pigments			Elemental chemistry					Nucleic acid			Growth
Factor		$rETR_{max}$	E_k	F_v/F_m	*Chl a*	*Chl c*	Fuco	%C	%N	C:N	$\delta^{13}C$	$\delta^{15}N$	RNA	DNA	RNA:DNA	Rel gr.
	df	11	11	11	12	12	12	12	12	12	12	12	12	12	12	12
Light (L)	2	**21.9*****	**5.22***	2.67	1.09	**4.93***	2.04	1.63	1.33	1.30	2.54	**35.0*****	0.67	**11.8****	2.47	2.58
Nitrate (N)	1	0.65	1.01	0.01	**4.78***	0.21	2.02	0.52	0.45	0.75	0.82	1.33	0.17	0.32	0.03	**13.3****
L x N	2	**5.70***	0.98	0.47	1.21	1.81	0.93	3.38	0.81	1.65	0.76	0.64	1.94	2.74	2.06	1.83
Tukey's																
Light (L)		HL>LL>ML	HL>ML			LL>ML						LL=ML>HL		HL>ML=LL		
Nitrate (N)					LN>HN											LN>HN
L x N		HL>ML @LN&HN HL>LL@HN														

Table 2.2 – F-test statistics for three-way factorial ANOVA testing for effects of temperature (3 levels: 12, 17 & 22 °C) except at T_{end} for nucleic acids, elemental chemistry and relative growth rate where there were 2 levels (12 & 17°C), light (2 levels: 6 & 30 µmol photons m^{-2} s^{-1}) and nitrate (2 levels: 0.5 & 3.0 µM L^{-1}) on Tasmanian-sourced juvenile *Macrocystis pyrifera* photosystem II, pigments, elemental chemistry and nucleic acids at T_0 A. and T_{end} B. Results of Tukey's HSD tests for significant results are given below the table. Only significant differences are shown (i.e. levels not included in table are not significantly different to all other levels). Abbreviations given in the Tukey test are: moderate light (ML); low light (LL); low nitrate (LN); high nitrate (HN). Degrees of freedom (*df*) for treatment effects (vertical) and denominator (horizontal); df = 2 (#: Table 2.2B only). Test of significance: *** P < 0.001; ** P < 0.01; * P < 0.05.

A		**PSII**			**Pigments**			**Elemental chemistry**					**Nucleic acid**		
Factor		$rETR_{max}$	E_k	F_v/F_m	*Chl a*	*Chl c*	Fuco	%C	%N	C:N	$\delta^{13}C$	$\delta^{15}N$	RNA	DNA	RNA:DNA
	df	19	19	24	24	24	24	24	24	24	24	24	23	24	23
Temperature (T)	2	**12.2*****	**31.7*****	**54.8*****	0.45	2.35	0.90	0.79	0.88	0.91	0.18	0.04	2.62	1.34	**3.66***
Light (L)	1	**12.5****	**8.65****	**21.0*****	0.81	**10.6****	0.34	**38.7*****	**10.5****	**29.9*****	**8.03****	0.02	1.53	**8.65****	**11.3****
Nitrate (N)	1	0.01	0.01	3.23	1.30	0.07	0.87	0.01	0.92	0.10	3.34	0.02	2.90	0.01	1.44
T x L	2	0.21	2.10	**23.5*****	0.43	0.19	0.73	0.06	**3.80***	3.06	1.15	1.27	**4.85***	0.18	2.00
T x N	2	2.26	**4.27***	3.07	0.01	1.28	0.29	0.65	0.53	0.36	1.59	0.02	0.35	0.07	0.02
L x N	1	0.68	0.05	3.67	0.04	0.25	0.01	0.37	0.01	0.10	0.44	1.27	**4.61***	1.11	0.10
T x L x N	2	1.21	2.05	1.83	0.82	0.13	0.44	1.43	0.05	0.50	0.13	0.26	0.98	0.25	0.51
Temperature (T)		17>12	17=22>12	12=17>22											22>17
Light (L)		ML>LL	ML>LL	ML>LL		LL>ML		ML>LL	LL>ML	ML>LL	LL>ML			ML>LL	LL>ML
T x L				12=17>22@LL ML>LL@22					LL>ML@22				22>12@LL LL>ML@22		
T x N			22>12 17>12 @HN&LN												
L x N													LN>HN@ML		

Table 2.2 (Cont'd)

B		**PSII**			**Pigments**			**Elemental chemistry**					**Nucleic acid**			**Growth**
Factor		$rETR_{max}$	E_k	F_v/F_m	*Chl a*	*Chl c*	Fuco	%C	%N	C:N	$\delta^{13}C$	$\delta^{15}N$	RNA	DNA	RNA:DNA	Rel gr.
	df	15	15	16	24	24	24	16	16	16	16	16	16	16	16	16
Temperature (T)	1#	**8.22***	**19.6*****	1.30	**3.76***	0.92	1.29	0.05	0.15	0.09	1.36	1.60	0.73	0.01	2.57	**9.15****
Light (L)	1	0.60	2.81	2.00	**27.6*****	**48.9*****	**3.46***	4.40	0.04	1.01	3.08	**18.8*****	1.36	1.52	3.08	2.82
Nitrate (N)	1	0.30	0.30	0.01	0.29	1.30	0.51	0.01	**7.24***	**6.65***	0.35	**5.04***	0.21	3.15	1.37	**4.75***
T x L	1#	**6.34***	0.73	0.09	2.71	0.77	0.53	0.02	6.45	5.18	1.02	**8.80****	0.27	1.05	2.61	2.68
T x N	1#	2.55	0.18	0.57	**4.61***	**7.10****	**4.06***	0.77	1.27	1.08	2.00	2.88	0.53	2.74	0.60	1.46
L x N	1	0.69	0.01	0.08	0.27	2.07	1.44	**7.38***	**9.61****	**15.1****	0.72	0.50	1.25	2.51	0.41	1.18
T x L x N	1#	2.31	0.57	0.19	3.35	2.05	1.90	0.73	1.68	0.36	0.50	2.90	0.65	0.64	0.05	3.07
Tukey's																
Temperature (T)		17>12	17>12		17>12											12>17=22
Light (L)					LL>ML	LL>ML						LL>ML				
Nitrate (N)									HN>LN	LN>HN		LN>HN				LN>HN
T x L		17>12 @ML only										LL>ML@17 17>12@LL				
T x N					22>12 @HN only	12>22 @LN only HN>LN @22 only	22>12 @HN only									
L x N								ML>LL @HN	HN>LN @LL	ML>LL @HN LN>HN @LL						

Table 2.3 – F-test statistics for two-way factorial ANOVA testing for effects of temperature (3 levels: 12, 17 & 22 °C) and nitrate (2 levels: 0.5 & 3.0 µM L^{-1}) at 30 µmol photons m^{-2} s^{-1} on Tasmanian-sourced juvenile *Macrocystis pyrifera* photosynthesis, pigments, elemental chemistry and nucleic acids at T_{end}. Results of Tukey's HSD tests for significant results are given below table. Only significant differences are shown (i.e. levels not included in table are not significantly different to all other levels). Abbreviations given in the Tukey test are: low nitrate (LN); high nitrate (HN). Degrees of freedom (*df*) for treatment effects (vertical) and denominator (horizontal). Test of significance: *** P < 0.001; ** P < 0.01; * P < 0.05.

END		**PSII**			**Elemental chemistry**					**Nucleic acid**			**Growth**
Factor		$rETR_{max}$	E_k	F_v/F_m	%C	%N	C:N	$\delta^{13}C$	$\delta^{15}N$	RNA	DNA	RNA:DNA	Rel gr
	df	10	10	11	12	12	12	12	12	12	12	12	12
Temperature (T)	2	**12.5*****	**5.46***	**13.3** ***	0.08	**28.1*****	**29.1*****	0.66	**9.08****	0.77	0.26	2.27	**16.4*****
Nitrate (N)	1	0.05	0.17	0.16	1.08	**5.49***	4.19	4.23	**5.76***	2.74	0.08	1.33	1.90
T x N	2	0.38	0.05	0.67	1.85	**7.45****	**12.6****	1.53	1.23	1.17	1.38	0.40	0.19
Tukey's													
Temperature (T)		17>(12=22)	17>12	12=17>22		12=17>22	22>12=17		22>12=17				12=17>22
Nitrate (N)						HN>LN			LN>HN				
T x N						12=17>22@LN HN>LN@22	22>12=17@LN LN>HN@22						

Table 2.4 – PERMANOVA results comparing the multivariate phenotype of *Macrocystis pyrifera* between orthogonal treatments of Temperature (12, 17 & 22 °C), Light (10 & 30 µmol photons m^{-2} s^{-1}), and Nitrate (0.5 & 3.0 µM NO_3^-) at T_0 *and* T_{end}. Interaction terms with negative coefficients of variation were dropped from the analysis.

	T_0				T_{end}			
Source of variation	**df**	**MS**	**Pseudo-F**	***P***	***df***	**MS**	**Pseudo-F**	***P***
Temperature (T)	2	821.33	3.3019	**0.002**	2	677.74	4.198	**< 0.001**
Light (L)	2	1786.5	7.1821	**< 0.001**	2	1118.1	6.926	**< 0.001**
Nitrate (N)	1	388.74	1.5628	0.183	1	514.88	3.189	**0.014**
T x L	2	570.49	2.2935	**0.036**	1	507.21	3.142	**0.010**
T x N	-	-	-	**-**	2	466.36	2.889	**0.008**
L x N	-	-	-	**-**	2	380.96	2.360	**0.016**
T x L x N	-	-	-	**-**	**-**	-	-	-
Residual	33	248.75			21	161.43		

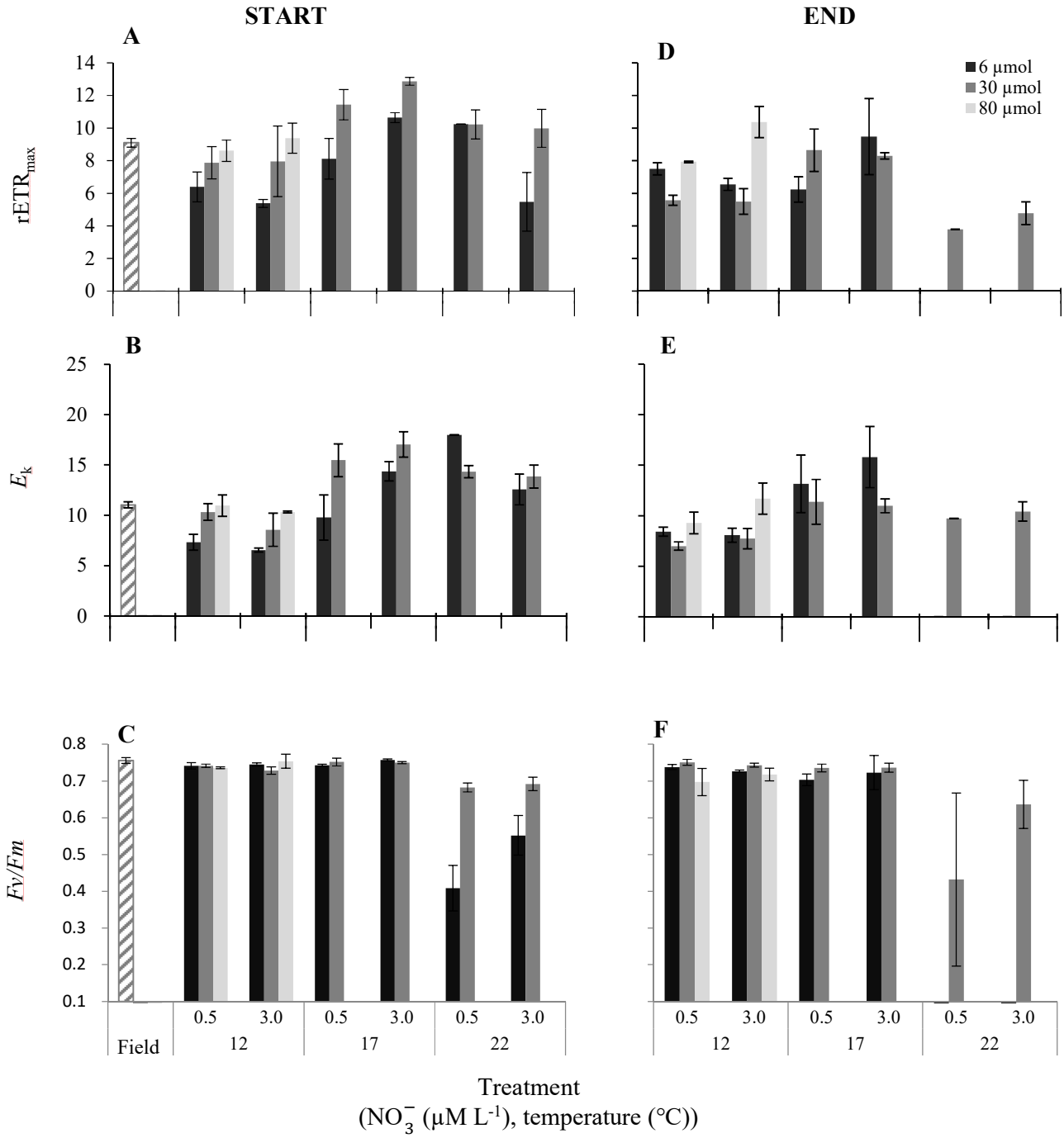

Figure 2.1 – Photosystem II (PSII) traits of *Macrocystis pyrifera* sporelings originating from Tasmania measured in situ and after exposure to experimental treatments of all combinations of temperature (3 levels), nitrate (2 levels), and light (3 levels – see legend) following acclimation to experimental conditions at T_0 (A-C), and after ≤ 28 days in experimental treatments T_{end} (D-F). Plots show (A, D) maximum relative electron transport rate ($rETR_{max}$); (B, E) saturating light intensity (E_k); (C, F) maximum quantum yield (F_v/F_m); as derived from dark-adapted RLCs measured by PAM fluorometry. Bars indicate mean values (n = 3) ± SE.

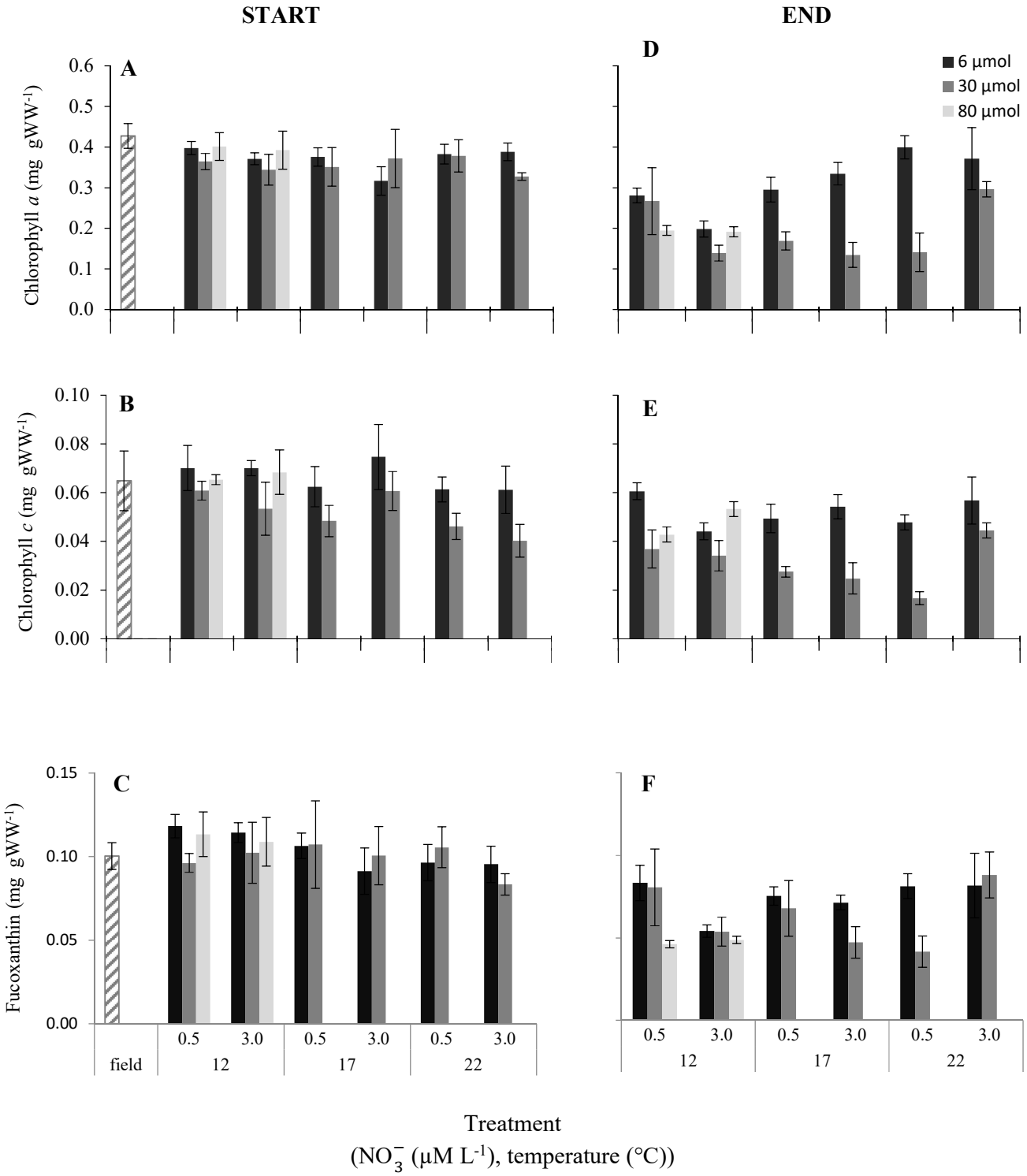

Figure 2.2 – Pigment content of *Macrocystis pyrifera* sporelings originating from Tasmania measured in situ and after exposure to experimental treatments of all combinations of temperature (3 levels), nitrate (2 levels), and light (3 levels – see legend) following acclimation to experimental conditions at T_0 (A-C) and T_{end} (D-F). Plots show tissue concentrations of (A, D) Chlorophyll *a*; (B, E) Chlorophyll *c*; (C, F) Fucoxanthin. Bars indicate mean values (n = 3) ± SE.

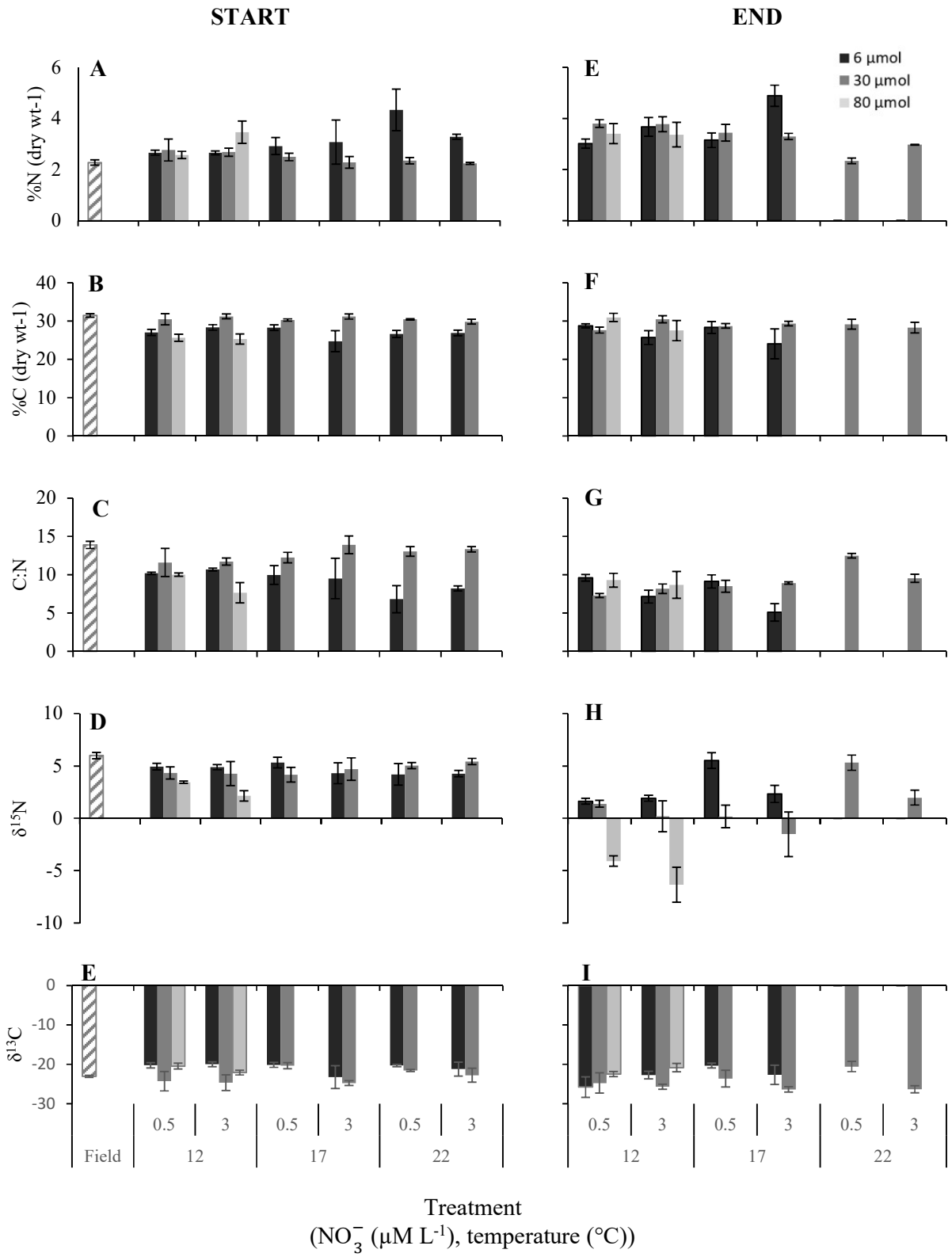

Figure 2.3 – Elemental chemistry of *Macrocystis pyrifera* sporelings originating from Tasmania measured *in situ* and after exposure to experimental treatments of all combinations of temperature (3 levels), nitrate (2 levels), and light (3 levels – see legend) following acclimation to experimental conditions at T_0 (A – E) and T_{end} (F – J). Plots show tissue proportions of carbon (A, F); nitrogen (B, G); carbon:nitrogen ratio (C, H); stable isotopic signature of $\delta^{13}C$ (D, I) and; $\delta^{15}N$ (E, J). Bars indicate mean values (n = 3) ± SE.

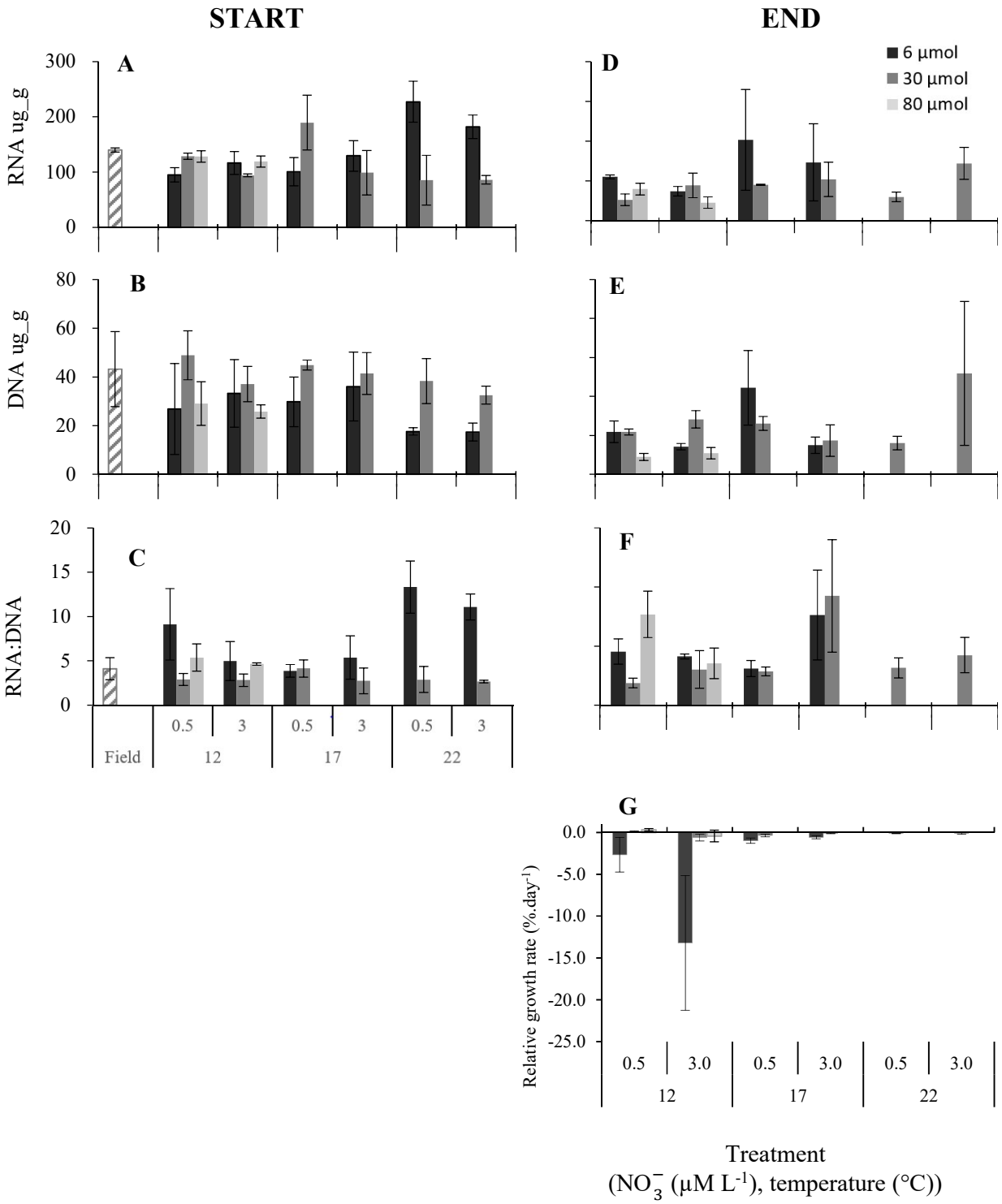

Figure 2.4 – Nucleic acid content and RNA:DNA rarios of *Macrocystis pyrifera* sporelings originating from Tasmania measured *in situ* and after exposure to experimental treatments of all combinations of temperature (3 levels), nitrate (2 levels), and light (3 levels – see legend) following acclimation to experimental conditions at T_0 (A – C) and T_{end} (D – F). Plots show tissue concentrations of (A, D) Chlorophyll *a*; (B, E) Chlorophyll *c*; (C, F) Fucoxanthin. Bars indicate mean values (n = 3) ± SE.

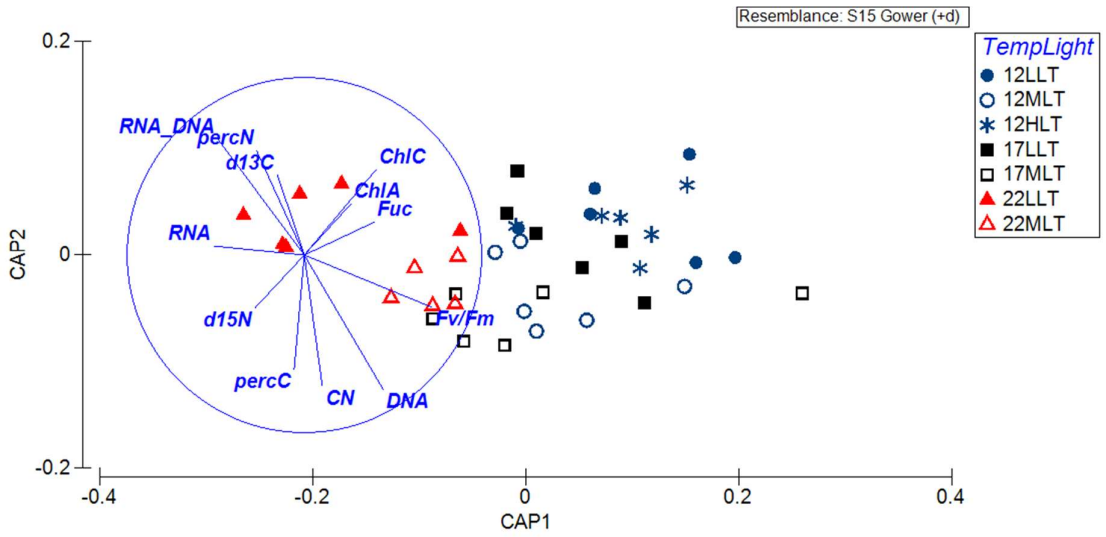

Figure 2.5 – CAP (Canonical Analysis of Principal coordinates) ordination of physiological response metrics of *Macrocystis pyrifera* to combined effects of temperature and light (based on a Gower similarity matrix of raw data for 12 traits) post-acclimation (T_0). The CAP analysis was constrained to differentiate among treatments of temperature and light levels, and shows clustering of light treatments and distinct separation of temperature effects. The vector overlay represents Pearson correlations between the ordination axes and direction and magnitude of trait influence. Abbreviations indicate: temperature (12, 17, 22 °C), light (low: LLT, medium: MLT).

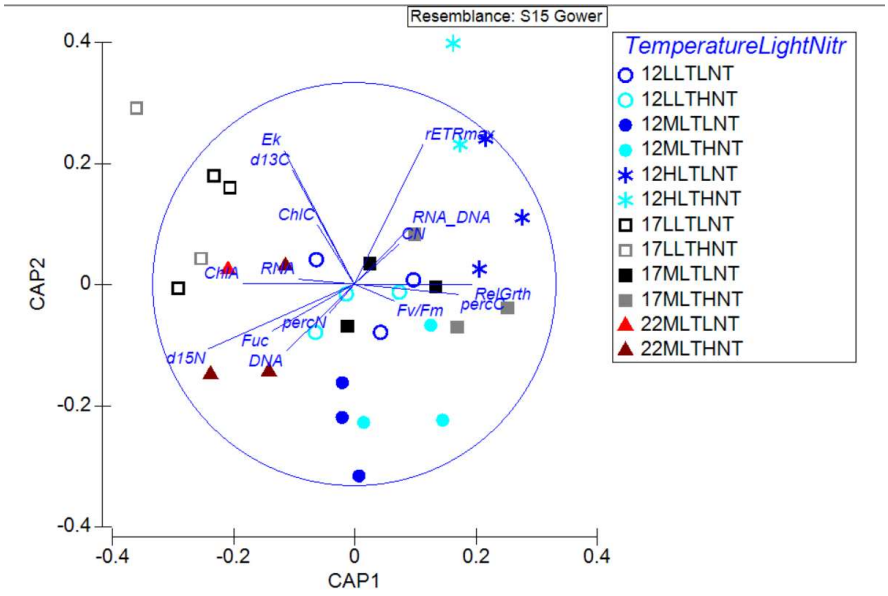

Figure 2.6 – CAP (Canonical Analysis of Principal coordinates) ordination of physiological response metrics of *Macrocystis pyrifera* to combined effects of temperature, nitrates and light (based on a Gower similarity matrix of raw data for 12 traits) after longer-term exposure to experimental treatments (T_{end}). The CAP analysis was constrained to differentiate among treatments of the different combinations of temperature, nitrate and light levels. The vector overlay represents Pearson correlations between the ordination axes and direction and magnitude of trait influence. Abbreviations indicate: temperature (12, 17, 22 °C), light (low: LLT, medium: MLT, high: HLT) and nitrate (low: LNT, medium: MNT, high: HNT).

2.5 Discussion

This study found that differential temperature, light, and nitrate levels lead to significant responses in a range of variables describing the physiology of *Macrocystis pyrifera* sporelings. Warming seawater negatively affected multiple traits in sporelings, consistent with previous studies in which elevated temperatures reduce growth and impair biological function in most temperate seaweeds (Harley *et al.*, 2012). Additionally, light and nitrates provided further impacts either independently, additively, or synergistically. These results illustrate the impacts on physiological processes that will likely shape the performance and distribution of *M. pyrifera* under future climate change in south eastern Australia.

2.5.1 Additive effects of temperature and light on M. pyrifera *condition*

Different temperature and light treatments caused significant variation in multiple physiological variables. Chronic macroscopic tissue deterioration and sporeling mortality in high temperature (22 °C) and high light (80 µmol photons m^{-2} s^{-1}) treatments were observed. Relationships between temperature and light and PSII are well documented for photoautotrophs, as PSII-associated enzyme catalysed reactions (RuBisCO and Calvin Cycle activity) are thermally labile (Davison & Davison, 1987; Raven & Geider, 1988; Wahid *et al.*, 2007) and electron transfer rates are light dependent (Ramus, 1981). This kind of change in enzymatic activity might explain the general pattern of increases in $rETR_{max}$ and E_k with increasing temperature (12 to 17 °C) and increasing light. Conversely, high temperature stress impairs biochemical pathways (Davison & Pearson, 1996) and can cause denaturation of proteins and degradation of thylakoid membrane properties (Blum & Ebercon, 1981; Maheswari *et al.*, 1999), inhibiting key photoprotective processes such as the production of chaperones (heat-shock proteins; Wahl et al. 2011). Moreover, nutrient uptake and gas exchange capabilities are impacted by high temperature causing downregulation of photosynthetic activity (i.e. a drop in $rETR_{max}$ and E_k), impairment of PSII (reduced F_v/F_m) (Heinrich *et al.*, 2012; Andersen *et al.*, 2013; Pereira *et al.*, 2015), and ultimately tissue

degradation and mortality as was observed at at 22 °C. Light energy absorption is independent of temperature and this energy is diverted into production of proteins, enzymes, and photoprotective mechanisms (Franklin *et al.*, 2003). Light absorbed in excess of photosynthetic demand generates reactive oxygen species that alter the permeability of chloroplast membranes and loss of electron transport capacity (Kyle *et al.*, 1984), disrupting carbon fixation and protein synthesis (Murata *et al.*, 2007), leading to chronic photoinhibition and photo damage where photo protection fails to mitigate photo inactivation (Franklin *et al.*, 2003; Hurd *et al.*, 2014a).

In the high-light treatments the increased energetic requirements of photosynthetic activity at temperatures above 12 °C may have undermined photoprotective capabilities, leading to rapid mortality in these treatment combinations. These findings highlight the vulnerability of *M. pyrifera* at temperatures above 12 °C under a reduced canopy where irradiance can fall within the range of 30 – 280 µmol photons m^{-2} s^{-1} in Tasmanian kelp forests (Tatsumi & Wright, 2016) and depends on depth, season and degree of canopy loss.

Conversely, in low light conditions investment into pigment synthesis can lead to an "energy crisis" where energy is diverted from carbohydrate and lipid production to producing light-harvesting pigments (Falkowski & LaRoche, 1991). In this context, the results suggest a synergistic interaction between temperature and light in that short term exposure (at T_0) to low light and high temperature at the same time quickly led to impaired PSII function (evidenced by low F_v/F_m) and significantly greater pigment content (the only determinable metric) in tissue subject to low light compared to moderate light. This suggests an increased vulnerability to ocean warming for *M. pyrifera* recruits in low light environments such as dense canopy understory, at depth, or locations subject to high sediment loads.

Elevated RNA:DNA ratios under high temperature and low light were also associated with impaired PSII (low F_v/F_m) after short-term exposure (T_0), and reflected both an overall increase in RNA concentration and a decrease in DNA levels in treatments with low light.

This association between PSII and nucleic acid levels may indicate elevated RNA synthesis of protective proteins in response to temperature-induced photosynthetic stress (as found in corals: Hauri et al. 2010), and/or low-light stress where down regulation of RuBisCO gene expression, transcription, and protein production is associated with lower cellular DNA concentrations, as has been identified in cucumber leaves (Sun et al. 2014). The nature of association between RNA:DNA and F_v/F_m found in *M. pyrifera* was contrary to that found in juvenile *Phyllospora comosa* (Flukes *et al.*, 2015), where a pre-condition for elevated RNA:DNA ratios was a healthy, functioning PSII (high F_v/F_m). These differences are possibly attributable to the extremely different light environments in which these species proliferate, with *P. comosa* occurring in much shallower waters than *M. pyrifera*.

In the context of the growth rate hypothesis (GRH) there was no evidence to support any relationship between RNA:DNA ratio and lineal growth relationship. This reflects the finding that ratios could change with changes in either the absolute concentration of DNA as found in *Phyllospora* (Flukes *et al.*, 2015), RNA or both. Variable DNA concentration is known in plants and seaweeds and can be influenced by cell packing density (Dortch *et al.*, 1983), increasing cell size or cell wall thickness (Kraemer & Chapman, 1991; Stirk *et al.*, 2011), and size of chloroplasts (Rauwolf *et al.*, 2010), characteristics that were undetectable by the measurements. Consequently, these results support that RNA:DNA ratio may a be more useful indicator of recent stress as RNA synthesises for stress-related protein complexes. Identification and quantification of particular stress proteins (i.e. heat-shock proteins) using qPCR methods could be used to indicate the type and severity of stress within populations.

2.5.2 Additive effects of temperature and nitrates on M. pyrifera growth

Sporeling growth rates were highest at low temperature and low nitrate levels. Lower growth rates at temperatures above 12 °C suggest a temperature 'growth boundary' (van den Hoek, 1982) between 12 and 17 °C for *M. pyrifera* in Fortescue Bay. Hence, it is probable that sustained positive temperature anomalies such as the recent 130-day heatwave event in south

east Australia (Oliver pers. comm.; Hobday et al. 2016) restrict opportunity for sporeling growth and development.

Annual variation of ocean nitrate levels in south eastern Australia generally follow a seasonal trend, ranging from ~0.5 μM NO_3^- and sometimes undetectable levels during the mid-late summer growth season to up to ~3.0 μM NO_3^- in the winter (Harris *et al.*, 1987; Sanderson, 1990). *M. pyrifera* and other kelps can actively regulate nitrogen uptake, assimilation, storage and use, and display locally adapted nitrogen utilisation strategies (Gagné *et al.*, 1982; Stephens & Hepburn, 2016), although relative to other Laminarian species *M. pyrifera* has poor capacity for nitrogen storage (Gerard, 1982, 1997). Interestingly, *M. pyrifera* sporelings in low nitrate treatments had higher relative growth rates, whilst those in high nitrate treatments displayed preference for uptake and storage of N (higher N concentrations and more negative $\delta^{15}N$ signatures) rather than growth *per se*. Sporelings with higher growth rates had lower nitrogen tissue concentrations indicating that nitrogen use outstripped replenishment rates, which is typical of Laminarians in low nitrate conditions (Gerard, 1982). However, this does not explain the observed lower growth rates in high nitrate treatments. Plastic response to stressors via the coupling of physiology and morphology is well documented in kelps (Druehl & Kemp, 1982; Fowler-Walker *et al.*, 2006). Resource acquisition influences morphology in macroalgae and nutrient stress (low ambient nutrients) may trigger a shift in resource allocation to thallus growth, increasing total surface area to volume ratio and improving nutrient uptake kinetics (Hein et al. 1995). Plants subjected to nutrient starvation can shift growth allocation from leaves to roots (Hirose & Kitajima, 1986) demonstrating resource-driven changes to morphology. An inverse relationship between nutrient availability and growth has also been described in unicellular marine algae where nitrogen deprivation leads to increased competition between carbon fixation and N assimilation (Hipkin *et al.*, 1983). This observation is contrary to results for Californian *M. pyrifera* where growth was limited at yearly minima (2 μM NO_3^-) but not limited at yearly

maxima (8 μM NO_3^-) in experimental conditions (Deysher and Dean 1986; Kopczak et al. 1991; Dayton et al. 1999). This distinction between different populations of *M. pyrifera* provides some evidence that nutrient utilisation dynamics in kelp can be locally adapted and that sporophyte development may be in part triggered by seasonal environmental cues, the timing of which has implications for sporophyte fitness (Kinlan *et al.*, 2003).

2.5.3 The future: efficacy of a multifactor approach

This study emphasises the importance of multifactor approaches to determining species and ecosystem response to climate change. Marine climate change studies up until recently have been predominantly temperature focused (Harley *et al.*, 2006) and have shown that temperature is clearly important for *M. pyrifera* and other marine species. However, this study demonstrates that effects of ocean warming may be substantially altered when there is simultaneous change in other environmental factors demonstrating the importance of multifactor approaches in climate change studies. Further to this, multivariate approaches to assessing physiology are crucial for holistic organismal-level insights into performance effects. Based on the lack of support for the GRH and the unexpected inverse relationship between relative growth and nitrogen treatment levels, it can be argued that predictions derived from simplified and generalised approaches such as RNA:DNA ratios and relative growth rates, may come with high levels of uncertainty (Schiel & Foster, 2015).

Yet a further layer of complexity is associated with 'ecosystem engineers' such as kelp whereby biotic structure modify the abiotic environment altering conditions for recruitment, which in turn may influence patch dynamics and stability (Dayton *et al.*, 1984; Jones *et al.*, 1994; Steneck *et al.*, 2002). It is likely that the treatment effects observed here will differ for adult and microscopic stages, as is the case in other species of brown algae (Wahl *et al.*, 2011). Hence, more research into the impacts of climate change on other stages is required to determine if and how populations may adapt. Future research must also consider genetic and non-genetic drivers that determine adaptability (Schiel and Foster 2016) such as genetic

diversity and phenotypic plasticity (Reusch, 2014). Nevertheless, sporelings are an important link in sustaining *M. pyrifera* populations and as demonstrated here, they exhibit negative responses and susceptibility across a range of physiological parameters. At the very least, temperature, canopy destabilisation (increased light), and disruption of nutrient regimes are certain to provide further distributional declines in *M. pyrifera*. Examining multiple physiological traits allows for a more comprehensive interpretation of the effects of climate change on overall physiology when compared to predictions based on one or a few types of metrics (i.e. chlorophyll, PSII).

Chapter 3 | Physiological response to increased temperature, reduced light and nitrates in the common kelp, *Ecklonia radiata* from two bioregions

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3.1 Abstract

Ecklonia radiata (J. Agardh) is a widespread habitat-forming kelp which dominates subtidal temperate reefs throughout southern Australia and forms critical habitat of the Great Southern Reef. The south-eastern region of the Reef is one of the most productive temperate reef zones in the world, yet is subject to temperature increases of four times the global average and oligotrophy associated with seasonal incursions of the East Australian Current. These stressors destabilise the kelp canopy (i.e. thinning), imposing light stress on juvenile kelp recruits (sporelings). The Growth Rate Hypothesis predicts warming and oligotrophic effects will be more severe for seaweed from higher latitudes as they have higher nutrient requirements than their lower latitude conspecifics. Lab experiments tested the effects of temperature (12 °C, 17 °C and 22 °C), light (10 & 30 µmol photons m^{-2} s^{-1}) and nitrate (0.5 and 3.0 µM NO_3^-) on multiple physiological factors (PSII, elemental chemistry, pigments, nucleic acids and growth) of the common kelp *Ecklonia radiata* sporelings from two distinct bioregions (New South Wales and Tasmania) over 29 days. Whilst temperature predominantly drove significant differences in physiology, seaweed from Tasmania and NSW responded differently to experimental treatments, and some main effects of light and nitrates, and higher-level interactions, emerged during the experiment.

3.2 Introduction

Predicting the impacts of climate change on ecosystems is a major challenge and underpins climate change adaptation. Understanding how environmental change will impact habitat-forming 'ecosystem engineers' is of particular importance as these species form the basis of hierarchically organised communities (Bruno & Bertness, 2001; Bruno *et al.*, 2003) and determine associated community structure and ecosystem function (Jones *et al.*, 1994, 1997). Globally, shallow subtidal temperate reefs are dominated by large habitat-forming seaweed, in particular kelp (Order Laminariales) which provide the foundation for diverse and productive ecosystems (Mann, 1973; Schiel & Foster, 1986; Steneck *et al.*, 2002). Thus, the ecophysiological response of kelp to environmental change is a key element in shaping subtidal temperate reef ecosystems into the future (Wernberg *et al.*, 2012b, 2016a).

Until recently, most studies of ecophysiological response of algae to climate change examined single factors, primarily temperature, and these typically show reduced performance at higher temperatures (Hatcher *et al.*, 1987; Serisawa *et al.*, 2002; Andersen *et al.*, 2013; Jueterbock *et al.*, 2014). However, because climate change involves multiple environmental factors, a more realistic approach is to examine the response to multiple factors simultaneously to identify the possibility of additive, synergistic, or antagonistic responses. For example, elevated temperature and CO_2 together significantly increase mortality rates in early-life history stages of *Macrocystis pyrifera* (Gaitán-Espitia *et al.*, 2014) and high CO_2 alters the effects of temperature, light and nutrients on physiology and productivity of turfing algae in the kelp understory (Connell & Russell, 2010a; Russell *et al.*, 2011; Falkenberg *et al.*, 2013). Additionally, given the underlying environmental gradients, biogeographic variation as a result of either phenotypic plasticity or evolutionary adaptation reflects physiological responses to environmental stress, with temperate seaweed (microscopic through to adult stages) from higher latitudes (cooler climates) typically

exhibiting a lower thermal tolerance (Novaczek, 1984; Flukes *et al.*, 2015; Pereira *et al.*, 2015). Thus, physiological response to climate change is expected to vary across latitudes, and one possibility is that it may be explained in part by the Growth Rate Hypothesis (GRH) (Elser *et al.*, 2003) which suggests that selection for higher growth rates at higher latitudes compensates for growth seasons shortened by lower temperatures and reduced light (Elser *et al.*, 2000b). Since growth depends on protein synthesis and P-rich ribosomal RNA, the GRH suggests that organisms with faster instantaneous growth rates (i.e. autotrophs at higher latitudes) will have higher concentrations of RNA and higher nutrient requirements (Elser *et al.*, 2003). If this holds, then seaweed at higher latitudes will have higher RNA tissue concentration and nutrient requirements, and thus be more susceptible to nutrient limitation. Empirical evidence supporting this idea exists for invertebrates and microalgae (Dortch *et al.*, 1983; Lepp & Schmidt, 1998; Elser *et al.*, 2000a; Lovelock *et al.*, 2007), and rapid-growing seaweed (Giordano *et al.*, 2015) but limited testing of this hypothesis exists for large, slow growing seaweeds (Reef *et al.*, 2012; Flukes *et al.*, 2015).

Southern Australia supports the Great Southern Reef as one of the most productive temperate reef zones in the world (Bennett *et al.*, 2016) but the south east region is warming at almost four times the global average (Johnson *et al.*, 2011), attributable to more regular seasonal southerly incursions of East Australian Current (EAC) waters (Ridgway & Hill, 2012). EAC influenced ocean warming is associated with oligotrophic, nutrient depleted waters, with levels typically <0.3 μM NO_3^- and often undetectable (Harris *et al.*, 1987) potentially exerting simultaneous abiotic stress on macroalgae that rely on seasonal nutrient loading, storage and assimilation for growth and other metabolic processes (i.e. *Laminaria longicruris* and *Ecklonia cava*: Gagné et al. 1982; Gao et al. 2016).

Ecklonia radiata (C. Agardh) J. Aghard is the dominant and most widespread canopy-forming seaweed of the Great Southern Reef. It is a perennial stipitate kelp that grows up to 2

m in length and forms a dense canopy. *E. radiata* dominates subtidal rocky reefs over a wide latitudinal range from warm to cool temperate waters and over a large depth range from 2 - > 60 m (Womersley, 1981; Marzinelli *et al.*, 2015). *E. radiata* habitat supports diverse food webs making it the most important marine habitat-forming species in southern Australia (Shepherd & Edgar, 2013; Bennett *et al.*, 2016). *E. radiata* displays a typical Laminarian life cycle consisting of microscopic male and female gametophytes that develop from zoospores released from sori on the adults, with juvenile sporophyte recruits (sporelings) developing from fertilised oogonia on female gametophytes. *E. radiata* sporelings have a propensity to grow and remain in sound physiological shape under the low light conditions of the adult canopy until, depending on the seasonal timing, canopy disturbance increases understory light exposure, leading to a rapid growth response in successful sporelings (Flukes *et al.*, 2014) but also up to 50% chance of mortality likely due to photoinhibition through light saturation and exposure (Toohey & Kendrick, 2007). In the relatively near future Tasmania's kelp forests may be subject to sea surface temperature increases of ~3.0°C (by 2100; Ridgway and Hill 2012) combined with extended periods of low nutrients (Rochford, 1984), increased heatwave events (Wernberg *et al.*, 2010, 2016a) and the proliferation of grazers (Ling *et al.*, 2009; Johnson *et al.*, 2011). These stressors will likely destabilise and thin kelp canopies causing changes in understory light regimes and recruitment patterns. Thus, predicting the combined impacts of variation in temperature, light, and nitrate concentrations on *E. radiata* populations is critical to predict the impact of future climate scenarios and assist in the management of these habitats.

The objective of this study was to explore climate change impacts on the physiology of juvenile *E. radiata* from two distinct latitudes, and in particular to evaluate the effects of temperature, nitrate, and light separately and in combination. Specifically, laboratory experiments examining the interactive effects of temperature, nitrate concentration, and light

on a range of traits in juvenile *E. radiata* from its northern and southern range to test the hypotheses: 1) that the effects of ocean warming, reduced nutrient availability, and reduced canopy cover (increased light) on *E. radiata* physiological performance are additive; and 2) that the performance of *E. radiata* from low latitudes (NSW) will not indicate a response to climate change in higher latitudes (Tasmania) due to adaptation of distinct populations and ecotypes.

3.3 Materials & methods

3.3.1 Collection and in situ measurements

E. radiata sporelings (50 – 150 mm blade length) were collected from the species' northern range at Port Stephens, New South Wales (NSW) (32.630594 °S, 152.309621 °E) in February 2012 and southern range at Fortescue Bay, Tasmania (43.123024°S, 147.976355°E) in October 2012 at a depth of ~10 m. At the time of collection, *in situ* water temperatures were ~20 °C and 12 °C respectively and surface photosynthetically active radiation (PAR) was ~9 mol photons m^{-2} day^{-1} and ~10 mol photons m^{-2} day^{-1} respectively. Sporelings were placed into an aerated cooler containing fresh seawater and held under low light (12:12 light & dark cycle; 12 µmol photons m^{-2} s^{-1}) and temperature (13-15°C) conditions for 36 hours to slow respiration and photosynthesis rates and reduce the risk of temperature, oxygen and nutrient stress (Peckol, 1983) during transportation back to the laboratory. Samples from both regions were treated identically, aside from air travel for the NSW samples only.

During collections, *in situ* baseline physiology was measured from four haphazardly selected sporelings at each location. PAM fluorometric measurements were taken and tissue was collected for pigment, other chemistry, and nucleic acid analysis (see *3.3.4 Physiological measurements).*

3.3.2 Experimental design and growth conditions

The response of Tasmanian and NSW *E. radiata* sporelings to temperature, irradiance and nitrates were determined using a three-way factorial laboratory experiment with the factors temperature (12, 17, 22 °C), irradiance (10, 30 µmol photons m^{-2} s^{-1}), and nitrate concentration (0.5, 3.0 µM NO_3^-). *E. radiata* sporelings from Tasmania and NSW were grown in separate trials for up to 29 days with three independent replicates for each temperature-irradiance-nitrate combination.

Upon arrival at the laboratory, sporophytes were divided between three holding tanks containing aerated 0.2 µm filtered seawater from the same source, shaded with layered flyscreen at 13-15°C, and assigned to temperature treatments. An earlier pilot trial indicated acute physiological stress in *E. radiata* sporelings under sudden increases in temperature and light. Consequently, incremental changes to temperature (2 °C day^{-1}) were made until the three experimental target temperatures were reached. After 5 days, thalli were haphazardly selected from the tubs and two individuals were placed into a glass beaker (2000 ml) containing growth media, with three independent beakers allocated to each treatment combination of temperature, light and nitrate. Thalli were free-floating, as a pilot study showed no difference in physiological performance between vertically oriented and free-floating thalli. Beakers were shaded by multiple layers of flyscreen to achieve an irradiance of 10 µmol photons m^{-2} s^{-1}. Once temperature conditions were achieved, thalli were light-acclimated for a further two days prior to initiating the growth experiment and irradiance was increased by removing layers of flyscreen by 10 µmol photons m^{-2} $s^{-1}.day^{-1}$ until the target level of 30 µmol photons $m^{-2}s^{-1}$, which represents an average for beneath the *Ecklonia* canopy (Tatsumi and Wright 2006), was reached. Media was replenished every 2-3 days for the duration of the experiment to ensure nutrient levels were maintained.

Growth media (Wright's Chu #10 (WC)) comprised autoclaved, nutrient depleted, 0.2 µm filtered seawater with added base stocks of vitamins, trace metals, potassium hydrogen sulphate (K_2HPO_4) and sodium nitrate ($NaNO_3^-$) (Guillard & Lorenzen 1972: reviewed by Andersen 2005). Nitrate levels (3.0 µM and 0.5 µM NO_3^-) were chosen based on historical (high) and EAC influenced (low) ranges observed at Maria Island and off the NSW coast (Rochford, 1984). Manipulation was achieved by modifying the volume of $NaNO_3^-$ added to WC media to achieve target concentrations, whilst keeping the ratio of phosphorus to nitrate stoichiometrically balanced at 20:1 by adding K_2HPO_4 (Guillard & Lorenzen, 1972) to avoid limiting of macronutrients. Aeration of the media in each beaker was used to promote circulation of media around the thalli, with air passing through a 0.2 µm syringe filter to minimise media contamination risk. Temperature-controlled rooms were set to 12, 17 and 22 °C. This range of temperatures was approximated due to the temperature range experienced by *E. radiata* at NSW and Tasmanian sites (Tasmania minima ~11°; NSW maxima ~23°C; Flukes *unpub. data*). Lighting consisted of 40 watt Sylvania standard cool white globes (model F40W/133-RS) on a 12:12 light : dark cycle, and selection of light levels was based on field measurements under kelp clearance experiments at Fortescue Bay where maximum PAR dose in January was ~9 and ~25 µmol photons m^{-2} s^{-1} in low and high density canopy treatments respectively (M. Tatsumi *unpub. data*).

3.3.3 Physiological measurements

Physiological responses were measured sacrificially from the pair of thalli in each beaker at the beginning (T = 0 days, where T_0 = end of acclimation period) and end of the experiment (T_{end}: up to 29 days after T_0). On both occasions, PAM fluorometry measurements were taken, then tissue was sacrificed for chlorophyll, tissue chemistry, and nucleic acid analysis. At T_0 photographs were taken of the second individual to record growth against photographs of the same individual at the end of the experiment. Once PAM fluorometry was conducted,

thalli were cut into sections of 10-20 mm^2 for nucleic acid analysis and stoichiometric analysis, and larger sections (20 - 40 mm^2) for pigment analysis (see below). All sections were handled with gloves, tweezers and a scalpel after being washed in dH_2O then patted dry and prepared for storage before processing. Tissue chemistry sections were immediately placed into a -20 °C freezer for further analysis and pigment sections were immediately frozen in darkness at -20 °C. Nucleic acid sections were placed in RNAlater (Ambion. Inc., Austin, Texas), a non-toxic stabilisation buffer that preserves total tissue RNA and DNA, and then frozen at -20 °C.

3.3.4 PAM fluorometry

Potential photosynthetic performance in juvenile *E. radiata* was estimated from rapid light curves (RLCs) obtained by measuring variable chlorophyll *a* fluorescence in photosystem II (PSII) as a function of PAR using a blue-light diving PAM fluorometer (Walz, Germany). To ensure consistency of fluorescence measurements and to eliminate ambient light interference, a leaf clip with a closable window was attached to thalli just above the meristematic region to maintain uniform spacing between the fibre optic light source and thallus tissue. RLCs were generated by an internal PAM software routine where actinic light intensity increased in eight steps of 10 seconds each, and at each step effective quantum yield of PSII (ϕ_{PSII}) and PAR was measured. Relative electron transport rate (rETR) was determined by multiplying ϕ_{PSII} by the respective PAR (Schreiber *et al.*, 1994), which estimates the rate of electrons pumped through the photosynthetic chain (Beer *et al.*, 2001). Estimates of electron transport dynamics were determined by plotting rETR against PAR and fitting to Platt et al's (1980) double exponential decay model (see below).

RLCs were determined at two adjacent locations on single thalli under pre-treatments of ambient light and dark-acclimation (approximately 15 minutes or more). Dark-acclimation duration was determined by pilot studies to be approximately 15 minutes, after which more

time under dark conditions showed no further increases in F_m or F_v/F_m, indicating re-oxidation of the electron transport chain and relaxation of the photoprotective mechanisms. Ambient light RLCs reflect the immediate light history and can be affected by ambient irradiance conditions (i.e. cloud cover, canopy shading, water turbidity, etc.), whereas dark-acclimated RLCs indicate the inherent state of the photosystem (Ralph & Gademann, 2005). PSII characteristics of marine autotrophs varies diurnally (Lorenzeen, 1963; Ramus & Rosenberg, 1980; Belshe *et al.*, 2007) and maximal performance of PSII tends to be symmetrical around a midday axis (Ramus & Rosenberg, 1980), thus all PAM fluorometry was conducted between the hours of 10:00 and 14:00. When conducting PAM fluorometry with a leaf clip, local areas of tissue are subject to saturating pulses of actinic light, so it was ensured that the two PAM measurements came from tissue ~2 cm apart for the light-acclimated and dark-acclimated readings.

RLCs can be described and compared by characterising the photosynthetic response (raw rETR data) as a function of light, determined by the initial linear response and the region of photoinhibition (Ralph & Gademann, 2005). Raw data (rETR) were fitted to Platt et al.'s (1980) double exponential decay function curve from which RLC parameters ($rETR_{max}$) and saturating light intensity (E_k) were estimated using equations provided in Ralph and Gademann (2005):

$$P = P_S\left(1 - exp\left(\frac{-\alpha E_d}{P_S}\right)\right) \times exp\left(\frac{-\beta E_d}{P_S}\right)$$

where P is the photosynthetic rate (rETR), α the initial slope before the onset of saturation, E_d is the incident downwelling irradiance of the PAM internal halogen light, β characterises the slope region where PSII declines after photoinhibition (Henley, 1993), and P_s is a scaling factor defined as the maximum potential rETR. Double exponential decay models (Platt *et al.*, 1980) were fit using a nonlinear least-squares function in the 'R' software environment (v

3.0.0) and parameters $rETR_{max}$ (maximum electron transport rate) and E_k were estimated using equations as per Ralph and Gademann (2005). To ensure convergence the regression model settings were as follows: iterations = 100; stepsize = 1/1024; tolerance = 0.00001; initial seed value for P = maximum rETR derived from raw data, α = slope of linear regression fitted to first three points of raw data (typically in the range 0.7-1.0).

The optimum quantum yield of PSII, (F_v/F_m) was determined from dark-acclimated measurements where the minimum (F_0) and maximum (F_m) fluorescence were used to calculate variable fluorescence (F_v), so that F_v/F_m could be estimated. F_v/F_m indicates the physiological capacity and PSII integrity of the measured tissue area in the absence of the ambient light environment.

3.3.5 Pigments

Frozen samples were thawed, patted dry and 100-150 mg of tissue was weighed (to the nearest 0.01 mg) and placed into a 15 ml vial containing 5 ml of N,N-Dimethylformamide (DMF: Sigma Aldrich Pty Ltd, Castle Hill, Australia) to facilitate pigment extraction. Extraction vials were pre-wrapped in aluminium foil and samples were processed rapidly in a fume hood under low light to avoid pigment damage from ambient light then placed in the freezer under total darkness at -20 °C for 96 hours for pigment extraction to occur.

3.3.5.1 Chlorophyll a and c

A 3 ml aliquot of pigment extract was pipetted into a centrifuge tube and centrifuged for 8 minutes at 8,000 rpm. Chlorophyll *a* and *c* content of the supernatant was determined spectrophotometrically (wavelengths: 664.5, 631 and 582 nm) using a Dynamica HALO RB-10 Spectrophotometer and processed using UV Detective software (v1.1). Blanks of 100% DMF were read every ten readings. In accordance with manufacturer's instructions, where absorbance read above 1.000, supernatant was diluted with DMF until absorbance dropped below 1.000. Dilution factor was recorded and factored into the final calculations for pigment

concentration. Chlorophyll *a* and *c* concentrations were calculated using the recommended absorption coefficients following Inskeep and Bloom (1985) and Seely et al. (1972).

3.3.5.2 Fucoxanthin

Fucoxanthin content was determined from the remaining ~2 ml extractant. Aliquots of 2 µl of extract were injected using Waters Acquity H-series Ultra High Performance Liquid Chromotography (UPLC, Waters, Milford MA, USA) coupled to a Waters Acquity Photodiode Array detector (PDA) with a Waters Acquity UPLC Ethylene Bridged Hybrid (BEH) C18 column (2.1 mm x 100 mm x 1.7 micron particles). Mobile phases comprised a gradient mixture of three solvents, prepared by Merck Chemicals (Merck KGaA, Darmstadt, Germany): acetic acid (1%), acetonitrile, and 80:20 methanol:hexane (Merck KGaA, Darmstadt, Germany). Initial conditions were held for 3.5 minutes in a 20% acetic acid solvent and 80% acetonitrile solvent, followed immediately by 80% acetonitrile and 20% methanol:hexane solvent for a further 5.5 minutes, followed by 3 minutes re-equilibration to original conditions. The column was held at 35° C and the flow rate was 0.35 ml min^{-1}. The PDA was monitored continuously over the range 230 to 500 nm. Under these conditions fucoxanthin eluted at 2.3 minutes. Initial calibration of the visible ultra-violet (UV-Vis) response at 440 nm for fucoxanthin was carried out on a freshly prepared standard solution (Sigma Aldrich, Castle Hill, Australia) made up at 1.26 µg ml^{-1} in methanol. Chromatograms were extracted at 440 nm from the raw data using Waters TargetLynx V4.1 software, and the area of the fucoxanthin peak was recorded and converted to mg ml^{-1} fucoxanthin before conversion to mg g^{-1} wet weight.

3.3.6 Elemental stoichiometry

Frozen samples were thawed, patted dry, freeze dried, and then weighed every 12-24 hours to 0.01mg until no further weight loss was detected and they were deemed anhydrous. Samples were ground and homogenised in a mortar and pestle and approximately 5 mg of powder was

placed into tin cups, which were folded gently prior to analysis. Carbon, nitrogen and isotope signatures ($\delta^{13}C$ and $\delta^{15}N$) were measured using Thermo Gas Chromatograph coupled to a Finnigan Mat Delta S isotope radio mass spectrometer in continuous flow mode at CSIRO, Hobart. Results were calculated as follows and are presented in standard sigma notation:

$$\delta^{13}C \text{ or } \delta^{15}N\ (‰) = \left[\frac{R_{sample}}{R_{standard}} - 1\right] \qquad \text{where R} = \frac{^{13}C}{^{12}C} \text{ or } \frac{^{15}N}{^{14}N}$$

Standards were replaced and run every 12 cycles with Pee Dee Belemnite (PDB) used as a standard for carbon and air as a standard for Nitrogen.

3.3.7 Nucleic acids

Absolute RNA and DNA was determined to obtain RNA:DNA ratios as a proxy for "growth potential" by extraction of total nucleic acids and RNA and DNA components from a single tissue sample. RNAlater preserved algae tissue weighing between 1-5 mg (w/w) was patted dry, weighed and homogenised in a round-bottom 2 µl centrifuge tube (Eppendorf Safe-lock microcentrifuge tube) using a drill pestle in an extraction buffer comprising 500 µl urea (4 M), 1% sodium dodecyl sulphate (SDS), trisodium citrate (1 mM), sodium chloride (0.2 M) and 5 µl of proteinase K (Urea/SDS buffer). To ensure stabilisation of nucleic acid, digestion of RNAses by Proteinase K and complete cell lysis, the homogenised solution was held at 37°C for 10 minutes then placed immediately onto ice. Impurities (e.g. chlorophyll, phenolic compounds, salts, detergents in urea/SDS) were removed by vortexing the solution with 750 µl of ammonium acetate, followed by centrifugation for 5 minutes at 14,000 RCF. The resulting supernatant was decanted into a 1.5 ml tube to which 700 µl isopropanol was added and the tube was gently inverted 40 times to aid complete mixing of total nucleic acids (tNA) and isopropanol. The total tNA was pelletised by centrifuge (10 mins at 14,100 RCF) and the pellet was washed twice in a 75% ethanol (EtOH) solution, then resuspended in 200 µl molecular grade H_2O at 55 °C for 10 minutes, and separated into two 100 µl aliquots.

To isolate RNA, a solution of 80 µl molecular grade water, 5 µl DNAse (DNase I – Biolabs M0303L) with 20 µl buffer (New England Biolabs - B0303S) was added to one aliquot for total DNA digestion, whilst 100 µl water and 5 µl RNAse (Sigma Aldrich - R6148-25ML) was added to the second aliquot to digest RNA for total DNA isolation. To facilitate digestion, aliquots were incubated at 37 °C for 20 minutes then stabilised on ice. Isolated nucleic acids were stabilised and extracted by vortexing (10 s) with 400 µl of urea/SDS buffer, vortexing (15 s) again, and centrifuging (10 min at 14,000 RCF) with 200 µl ammonium acetate (7.5M), decanting supernatant into 1.5ml tubes and binding and pelletising with isopropanol as described in the previous step. RNA and DNA pellets were washed twice in 75% EtOH and resuspended into 100 µL of molecular grade water (RNA) and EB buffer (DNA). RNA and DNA concentrations were measured by fluorescence assays using a Quibit assay probe and fluorometer and expressed as total RNA and total DNA (ug.g^{-1} wet weight tissue). These values were used to calculate the RNA:DNA ratios.

3.3.8 Growth

Absolute growth and relative growth rates were determined by measuring linear extension of the thallus blade to the nearest mm. Whole thalli were photographed on laminated 1 mm scaled paper taken at the beginning and end of the experiment and images were processed using the software ImageJ (v1.46). Daily relative growth rate was calculated as $R = \frac{\log_{e\,2} L - \log_{e\,1} L}{{}_2T - {}_1T}$ (Evans, 1972), where L is length in mm and T is time in days.

3.3.9 Statistical analyses

We compared sporeling 'baseline' physiology variables across the two populations at the time of collection using student's t-tests for individual parameters. Baseline multivariate physiology was explored using one-way PERMANOVA and principal coordinates analysis (PCO: to represent the data graphically) on Gower similarity matrix (Gower, 1971) generated from untransformed data. Three-way factorial ('temperature', 'light' and 'nitrate') univariate

Analysis of Variance (ANOVA) were used to test the significance of treatments at the beginning (T_0) and end of the experiment (T_{end}) on photo-physiology ($rETR_{max}$, E_k, F_v/F_m), pigment content (chlorophyll *a*, *c* and fucoxanthin), elemental chemistry (%C, %N, C:N, $\delta^{13}C$ and $\delta^{15}N$), and nucleic acids (absolute RNA & DNA, and RNA:DNA ratio). Tasmania and NSW experiments were analysed separately, as it was not possible to separate the effects of origin and season. ANOVA assumptions were checked and transformations determined using the Box-Cox method (Box & Cox, 1964). Where significant differences were found between treatments of more than two groups, Tukey's HSD were used to perform *a posteriori* multiple comparisons. Multivariate physiology was analysed using three-way permutational multivariate ANOVA (PERMANOVA; Anderson 2001) at both T_0 and T_{end} to determine overall treatment effects on joint distributions of response variables. Resemblance matrices derived from Gower similarity coefficients (Gower, 1971) were generated from raw data and PERMANOVA was conducted with 9999 permutations to calculate pseudo *F*-statistics. Terms with negative estimates of components of variation were pooled (Anderson *et al.*, 2008).

3.4 Results

3.4.1 Baseline field measurements

One-way PERMANOVA and PCO revealed significantly different multivariate physiology of *Ecklonia radiata* between sites (*Pseudo-*$F_{1,6}$ = 10.84, p = 0.031) (Fig. 3.1A). Minimum saturating irradiance (E_k), C:N ratio and DNA concentration were significantly greater in seaweeds from NSW (Table 3.1; Fig. 3.1). In contrast, Tasmanian sporelings had significantly more accessory pigments (chlorophyll *c* and fucoxanthin), a greater nitrogen composition and RNA:DNA ratios than NSW seaweeds (Table 3.1; Fig. 3.1). Seaweed at both sites had similar capacity for photosynthesis ($rETR_{max}$), optimum quantum yield (F_v/F_m), chlorophyll *a* and carbon concentrations (%C), carbon and nitrogen isotopic signatures ($\delta^{13}C$ and $\delta^{15}N$) and RNA concentration. F_v/F_m was within the 'healthy' range, affirming the integrity of PSII and overall tissue condition for use in further analyses.

3.4.2 Tasmanian E. radiata: short-term response to acclimation

Following acclimation (i.e. at T_0), rapid physiological response occurred in some variables while others showed no change. $rETR_{max}$ and E_k significantly increased with each increasing step in temperature while F_v/F_m was greatest at 12 °C compared to higher temperature groups (Fig. 3.2; Table 3.2A). There were no treatment effects on pigments over this period (Fig. 3.3) but temperature significantly influenced elemental chemistry with a higher fraction of $\delta^{15}N$, an increase in total carbon and reduction in total nitrate (hence a higher C:N ratio) at 12 °C compared to 22 °C. The heavy carbon isotope fraction $\delta^{13}C$ was greater (less negative) at low nitrates under high irradiance (light x nitrate interaction) (Fig. 3.4; Table 3.2A). While no significant short-term changes were observed in RNA at T_0, significant temperature effects on DNA concentration and RNA:DNA ratios were dependent on light and nitrate levels (i.e. significant temperature x light x nitrate interactions, Fig. 3.5; Table 3.2A).

3.4.3 Tasmanian E. radiata: longer term responses

3.4.3.1 Photosynthesis and pigments

While temperature continued to have a similar longer term effect on PSII characteristics ($rETR_{max}$ and E_k) by T_{end}, a significant temperature x nitrate interaction developed for most pigment metrics. At 17 °C all tissue pigments were more concentrated when grown in low nitrates than in high nitrates, and chlorophyll *c* concentration was significantly greater at 17 °C than at 22 °C when grown under low nitrate conditions (Table 3.2B, Fig. 3.3). Fucoxanthin concentration was significantly lower in low light conditions (Table 3.2B; Fig. 3.3).

3.4.3.2 Growth, nucleic acids and tissue chemistry

Tissue necrosis occurred in some sporelings, leading to loss of biomass. In excessive cases, early termination of ten individuals across the experiment was required to ensure adequate tissue for the physiological measurements. Early termination was most frequent at 22 °C, with five individuals sacrificed within 12 days. A further five individuals were terminated between 21-26 days (from both 22 and 17 °C treatments) and the remainder survived up until 29 days. Isotopic signatures were significantly different between temperature-light ($\delta^{13}C$) and temperature-light-nitrate ($\delta^{15}N$) interaction groups yet with comparatively small mean squares in contrast to main terms of temperature and light which (Table 3.2B; Fig. 3.4). There were no significant effects for other elemental variables. At T_{end}, the RNA:DNA ratio was significantly greater at 22 °C than at 17 °C, in-part driven by significantly lower absolute DNA concentrations at 22 °C than at other temperatures (Fig. 3.5; Table 3.2B). Relative growth was significantly greater at 12 °C compared to higher temperatures (Fig. 3.5; Table 3.2B).

3.4.3.3 Multivariate phenotype response to acclimation and longer-term exposure

Three-way PERMANOVA revealed significant variation in multivariate physiological response of juvenile *E. radiata* from Tasmania at both T_0 and T_{end} driven by main effects of temperature (T_0: Pseudo-$F_{2,29} = 7.36$, $p < 0.001$; T_{end}: Pseudo-$F_{2,25} = 4.94$, $p < 0.001$) but not light (T_0: Pseudo-$F_{1,29} = 1.73$, $p = 0.15$; T_{end}: Pseudo-$F_{1,25} = 2.37$, $p = 0.056$), nitrate (T_0: Pseudo-$F_{1,29} = 1.17$, $p = 0.32$ T_{end}: Pseudo-$F_{1,25} = 0.50$, $p = 0.73$) or any interaction terms (Table 3.4). At both times, *a priori* tests revealed all temperature groups were significantly different from one another.

3.4.4 NSW E. radiata: short-term response

Significant differences among treatments for most physiological measurements were evident at the end of the acclimation phase at T_0, except for chlorophyll *c*, RNA concentration and F_v/F_m. $rETR_{max}$ and E_k increased significantly with temperature; chlorophyll *a* and fucoxanthin concentrations were similar at 12 °C but lower at 22 °C compared to 17 °C, and chlorophyll *a* concentration increased under low light conditions (Table 3.3A; Fig. 3.7). Nitrogen concentration was significantly lower at 22 °C compared to 12 °C; temperature effects on carbon concentration were mediated by light and nitrate (significant three-way interaction), however, the interaction mean squares were comparatively low in contrast to temperature where C concentration decreased with increasing temperature. C:N ratio was significantly higher under low nitrate conditions, whilst $\delta^{15}N$ fraction was higher (less negative) under high nitrates and low irradiance (Table 3.3A; Fig. 3.8). RNA:DNA ratios were significantly lower at 22 °C compared to other temperature groups. Significant effects of temperature on DNA concentration were moderated by light and nitrates (three-way interaction) where, under certain light and nitrate conditions, higher values for DNA concentration occurred at high temperatures (22 °C) compared to low temperatures (12 °C) (Table 3.3A; Fig. 3.9).

3.4.5 NSW E. radiata: longer term responses

3.4.5.1 Photosynthesis and pigments

At T_{end}, no significant effects were detected for F_v/F_m, for which values ranged 0.7 – 0.8, nor were there any for chlorophyll *a* and *c* (Table 3.3B). The other PSII measures ($rETR_{max}$ and E_k) increased with temperature, while E_k also increased with nitrates (Table 3.3B; Fig. 3.6). $rETR_{max}$ increased with light (under high nitrate only) and nitrate (under high light only) (i.e. light x nitrate interaction), in addition, $rETR_{max}$ was significantly higher under high light – high nitrate treatment. Fucoxanthin concentration was higher at 17 °C compared to 22 °C (Table 3.3B; Fig. 3.7).

3.4.5.2 Growth, nucleic acids and tissue chemistry

Due to excessive tissue necrosis three individuals from 22 °C and one individual from 12 °C treatments were sacrificed early (at 13 and 19 days respectively) to ensure sufficient tissue for analysis. The remaining sporelings survived until T_{end} (29 days). Although no significant effects were detected for N concentration, C:N ratio, C isotope signature and absolute DNA; RNA:DNA ratios were significantly greater at 12 °C compared to higher temperatures. Variation in mean RNA concentrations showed a significant three-way interaction, however, mean squares for factor temperature was 'stand-out' compared to the interaction term, where mean RNA concentrations among temperature groups was highest in 12 °C treatments. In addition, the highest RNA concentration occurred at 22 °C, low light - high nitrate treatment combination (Table 3.3B; Fig. 3.9). There were no temperature effects on tissue chemistry measurements or relative growth at T_{end}, however high light resulted in significantly greater relative growth rates and significantly lower $\delta^{15}N$ signature values (i.e. greater tissue concentration of the growth media N); $\delta^{15}N$ signature values were also low in individuals grown at high nitrate levels (Table 3.3B; Fig. 3.8). In low light, C concentration was

significantly less under high nitrates, reflected by a significant light x nitrate interaction (Table 3.3B; Fig. 3.8).

3.4.5.3 Multivariate phenotype response

Three-way PERMANOVA revealed significant variation in multivariate physiological response of *E. radiata* sporelings from NSW at both T_0 and T_{end} to main effects of temperature (T_0: Pseudo-$F_{2,27}$ = 10.3, $p < 0.001$; T_{end}: Pseudo-$F_{2,27}$ = 5.88, $p < 0.001$), light (T_0: Pseudo-$F_{1,27}$ = 2.55, $p = 0.04$; T_{end}: Pseudo-$F_{1,27}$ = 5.15, $p = 0.001$) and nitrate at T_{end} but not T_0 (T_0: Pseudo-$F_{1,27}$ = 1.32, $p = 0.26$ T_{end}: Pseudo-$F_{1,27}$ = 2.48, $p = 0.037$) nor any interaction terms (Table 3.5). At both times, *a priori* tests revealed all temperature groups were significantly different from one another.

Table 3.1 – Baseline field means for NSW and Tasmania *E. radiata*. P-values generated by Student's t-tests for significant differences.

Factor	***NSW***		**TAS**		**t**	***P***
	mean	SE	mean	SE		
$rETR_{max}$	65.24	5.588	22.24	1.954	2.415	0.052
E_k	99.28	17.04	27.78	1.977	**-3.708****	0.010
F_v/F_m	0.759	0.010	0.719	0.031	1.846	0.114
Fuco	0.289	0.045	0.746	0.080	**4.975****	0.003
Chl *a*	0.639	0.068	0.914	0.145	-1.714	0.137
Chl *c*	0.026	0.018	0.100	0.020	**2.675***	0.037
$\delta^{15}N$	4.346	0.187	3.030	1.226	1.042	0.338
$\delta^{13}C$	-21.29	1.403	-23.33	1.294	1.077	0.323
%N	1.605	0.092	4.166	0.669	**3.836****	0.009
%C	26.40	1.121	21.98	2.391	1.673	0.145
C : N	16.51	0.437	6.249	2.053	**-4.857****	0.003
RNA	3.722	0.630	3.197	0.133	0.816	0.446
DNA	0.527	0.016	0.252	0.038	**-6.627*****	0.000
RNA : DNA	7.136	1.359	13.53	2.039	**2.608***	0.040

Tests of significance: *** $P < 0.001$; $P < 0.01$; $P < 0.05$

Table 3.2 – F-test statistics for three-way factorial ANOVA testing for effects of Temperature (3 levels: 12, 17 & 22 °C), Light (2 levels: 10 & 30 µmol photons m^{-2} s^{-1}) and Nitrate (2 levels: 0.5 & 3.0 µM NO_3^-) on Tasmanian-sourced juvenile *Ecklonia radiata* photosynthesis, pigments, elemental chemistry and nucleic acids at T_0 of experiment – A; and at T_{end} of experiment – B. Results of Tukey's HSD tests for significant results are given below table. For pairwise interaction terms, only significant differences are shown in the table (i.e. interaction levels not included in table are not significantly different to other levels). Error *df* = 24 for all tests unless specified. Abbreviations: ^ - error *df* = 23. Test of significance: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

A		**PSII**			**Pigments**			**Elemental chemistry**^					**Nucleic acid**		
Factor	*df*	$rETR_{max}$	E_k	F_v/F_m	*Chl a*	*Chl c*	*Fuco*	%C	%N	C:N	$\delta^{13}C$	$\delta^{15}N$	RNA:DNA	RNA	DNA
Temperature (T)	2	**45.7*****	**61.5*****	**19.9*****	0.07	0.01	0.38	**8.47****	**5.37***	**6.50****	0.27	**8.38****	**3.94***	0.36	**4.79***
Light (L)	1	1.40	2.78	0.05	2.84	2.60	2.68	0.00	0.14	0.07	2.22	0.07	0.08	3.34	2.60
Nitrate (N)	1	0.18	0.01	0.99	0.15	0.01	0.08	1.15	2.47	2.19	**5.34***	0.46	1.76	0.11	0.52
T x L	2	0.84	0.52	0.24	0.26	0.09	0.33	0.48	1.40	1.05	0.84	0.61	2.33	1.29	0.52
T x N	2	3.11	2.27	0.24	1.20	1.11	0.29	1.20	0.68	0.78	0.89	0.46	2.68	0.41	0.56
L x N	1	0.04	0.18	1.20	0.35	0.44	0.16	1.19	2.80	2.68	**6.21***	2.83	2.13	0.01	1.75
T x L x N	2	0.46	0.41	1.24	0.10	0.02	0.99	0.94	0.29	0.34	0.11	0.46	**3.80***	2.52	**4.93***
Tukey's															
Temperature (T)		22>17>12	22>17>12	12>17=22				12>22	22>12	12>22		12>22	12>22		22>12
Nitrate (N)											0.5>3.0				
L x N											0.5>3.0@30 30>10@0.5				
T x L x N													12>22@10;3.0		22>12@30:3.0 22>17@10:0.5 3.0>0.5@17:10 30>10@17:3.0

Table 3.2 (Cont'd)

B		**PSII**			**Pigments**			**Elemental chemistry^**					**Nucleic acid^**			**Growth**
Factor	*df*	$rETR_{max}$	E_k	F_v/F_m	*Chl a*	*Chl c*	*Fuco*	%C	%N	C:N	$\delta^{13}C$	$\delta^{15}N$	RNA:DNA	RNA	DNA	Rel gr.
Temperature (T)	2	**26.0*****	**18.7*****	**16.8*****	1.52	2.45	0.39	0.15	1.06	0.69	**9.49*****	**16.9*****	**3.67***	0.07	**5.41***	**3.47***
Light (L)	1	0.06	1.06	1.07	0.95	0.36	**6.71***	0.00	1.33	1.15	**8.46****	**110*****	1.46	0.00	1.01	2.36
Nitrate (N)	1	0.05	0.24	0.99	**9.42****	1.42	**5.41***	0.16	0.10	0.06	1.30	**8.43***	0.01	0.55	1.72	1.21
T x L	2	1.33	0.19	**3.54***	2.23	1.83	0.59	0.29	0.47	0.38	**4.35***	**4.64***	1.68	0.56	1.62	0.35
T x N	2	0.94	0.36	**5.36***	**7.59****	**8.90****	**6.76****	0.84	0.66	0.71	0.69	**6.68****	1.07	0.84	5.00	0.18
L x N	1	3.72	2.48	2.68	0.83	0.26	0.07	0.14	0.56	0.59	1.50	1.22	0.90	0.02	1.49	0.42
T x L x N	2	1.71	1.90	3.08	1.74	2.70	1.03	1.56	1.48	1.62	1.87	**3.71***	3.40	0.88	1.80	0.78
Tukey's																
Temperature (T)		22>17>12	22>17=12	12>22=17							22=12>17	22=17>12	22>17		12=17>22	12>17=22
Light (L)							30>10				30>10	10>30				
Nitrate (N)					0.5>3.0		0.5>3.0					3.0>0.5				
T x L				12>22@10 12>17@10&30							30>10@12 12>17@30 22>17@10	22=17>12@30 10>30@All Temps				
T x N				12>22@ 3.0 & 0.5 12>17@3.0	0.5>3.0 @17	0.5>3.0@17 17>22@0.5	0.5>3.0 @17					22>17>12@0.5 3.0>0.5@17				
T x L x N												22=17>12@30:0.5 10>30@12:0.5 3.0>0.5@12:30				

Table 3.3 – F-test statistics for three-way factorial ANOVA testing for effects of Temperature (3 levels: 12, 17 & 22 °C), Light (2 levels: 10 & 30 µmol photons m^{-2} s^{-1}) and Nitrate (2 levels: 0.5 & 3.0 µM NO_3^-) on NSW-sourced juvenile *Ecklonia radiata* photosynthesis, pigments, elemental chemistry and nucleic acids at T_0 of experiment – A; and at T_{end} of experiment – B. Results of Tukey's HSD tests for significant results are given below table. Only significant differences are shown in the table (i.e. levels not included in table are not significantly different to all other levels of main factor or interaction). Error *df* = 24 for all tests. Test of significance: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

A		**PSII**			**Pigments**			**Elemental chemistry**					**Nucleic acid**		
Factor	*df*	$rETR_{max}$	E_k	F_v/F_m	*Chl a*	*Chl c*	*Fuco*	%C	%N	C:N	$\delta^{13}C$	$\delta^{15}N$	RNA:DNA	RNA	DNA
Temperature (T)	2	**119*****	**140*****	3.23	**3.95***	0.02	**4.94***	**42.6*****	**10.39*****	0.19	0.03	0.37	**5.67****	1.76	1.86
Light (L)	1	1.64	0.61	0.33	**8.59****	3.00	3.34	2.52	0.46	0.93	2.11	**9.95****	2.73	0.62	0.61
Nitrate (N)	1	1.93	2.14	2.59	1.71	0.57	0.10	1.52	2.07	**4.85***	0.55	**6.22***	0.74	1.98	0.21
T x L	2	0.95	1.60	1.11	0.64	0.97	1.07	1.99	0.25	1.20	2.13	2.76	1.04	0.15	0.44
T x N	2	0.10	0.23	0.19	0.09	0.15	0.06	1.06	0.82	0.63	**4.57***	0.37	1.69	0.21	0.99
L x N	1	1.41	0.36	0.37	0.18	0.06	0.24	0.01	0.99	0.92	0.71	1.02	0.06	0.02	0.10
T x L x N	2	1.67	2.21	0.53	0.08	0.51	0.80	**3.96***	0.36	0.40	1.85	1.90	0.53	2.45	**3.78***
Tukey's															
Temperature (T)		22>17>12	22>17>12		17>22		17>22	12>17>22	12>22				12=17>22		
Light (L)					30>10							10>30			
Nitrate (N)										0.5>3.0		3.0>0.5			
T x N											12>17@3.0 3.0>0.5@17				
T x L x N								12>22 12>17@30:0.5 17>22@10:0.5							22>12@10:3.0 22>12@30:0.5 10>30@22:3.0

Table 3.3
(Cont'd)

B

		PSII			Pigments			Elemental chemistry^					Nucleic acid			Growth
Factor	*df*	$rETR_{max}$	E_k	F_v/F_m	*Chl a*	*Chl c*	*Fuco*	%C	%N	C:N	$\delta^{13}C$	$\delta^{15}N$	RNA:DNA	RNA	DNA	Rel gr.
Temperature (T)	2	**36.2*****	**48.4*****	0.52	2.18	2.71	**3.76***	2.38	1.55	1.42	0.01	0.24	**3.69***	**13.2*****	1.06	0.30
Light (L)	1	1.09	0.06	3.49	1.70	0.01	2.65	0.16	0.25	0.91	3.87	**29.5*****	0.46	0.53	3.54	**7.92****
Nitrate (N)	1	3.79	**10.2****	0.07	1.82	1.57	2.89	**6.47***	1.33	0.17	2.40	**11.5****	0.23	3.32	3.91	0.01
T x L	2	0.34	0.33	0.29	0.75	2.24	1.15	0.43	0.28	0.56	0.15	2.50	0.70	3.17	0.57	0.49
T x N	2	0.95	0.95	1.08	0.12	0.77	0.30	2.01	1.78	0.66	0.16	0.18	0.75	**4.19***	0.49	0.78
L x N	1	**8.16****	1.45	0.04	0.25	0.01	1.53	**4.54***	2.12	0.53	0.81	0.29	0.98	0.05	0.84	0.11
T x L x N	2	0.18	1.91	1.95	0.21	0.05	0.31	0.11	0.80	0.19	0.72	1.16	0.94	**5.43***	1.23	0.44
Tukey's																
Temperature (T)		22>17>12	22>17>12				17>22						12>17=22	12>17=22		
Light (L)												10>30				30>10
Nitrate (N)			3.0>0.5					0.5>3.0				0.5>3.0				
T x N														12>17=22@0.5 3.0>0.5@22		
L x N		30>10@3.0 3.0>0.5@30						0.5>3.0 @10								
T x L x N														12>22@10:0.5 22>17@10:3.0 10>30@22:3.0 3.0>0.5@22:10		

Table 3.4 – Results of PERMANOVA testing the effects of temperature (3 levels: 12, 17 and 22), light (2 levels: 10 & 30 µmol photons m^{-2} s^{-1}) and nitrates (2 levels: 0.5 & 3.0 µmol NO_3^-) on multivariate phenotype of *Ecklonia radiata* juveniles from Tasmania at T_0 and T_{end}. Tests of significance: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. Terms with negative coefficients of variation were dropped from the analysis.

	T_0			T_{end}		
Source	**df**	**MS**	**Pseudo-*F***	**df**	**MS**	**Pseudo-*F***
Temperature (T)	2	2323	**7.367*****	2	1052	**<0.001*****
Light (L)	1	544.8	1.728	1	504.5	0.056
Nitrate (N)	1	367.6	1.166	-	-	-
T x L	-	-	-	2	233.4	0.369
T x N	-	-	-	2	415.3	0.072
L x N	1	712.0	2.258	-		-
T x L x N	-	-		2	392.0	0.089
Residual	23	315.3		23	213.0	

Table 3.5 – Results of PERMANOVA testing the effects of temperature (3 levels: 12°C, 17°C and 22°C), light (2 levels: 10 & 30 µmol photons m^{-2} s^{-1}) and nitrates (2 levels: 0.5 & 3.0 µmol NO_3^-) on multivariate phenotype of *Ecklonia radiata* juveniles from NSW at T_0 and T_{end}. Tests of significance: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. Interaction terms with negative coefficients of variation were dropped from the analysis.

	T_0			T_{end}		
Source	df	MS	Pseudo-*F*	df	MS	Pseudo-*F*
Temperature (T)	2	2497	**10.32**	2	1249	**5.882*****
Light (L)	1	617.7	2.553	1	1094	**5.147****
Nitrate (N)	1	321.8	1.329	1	525.9	**2.475***
TxL	-	-	-	2	227.6	1.071
TxN	2	242.2	1.000	-	-	-
LxN	-	-	-	1	337.2	1.587
TxLxN	2	329.4	1.361	-	-	-
Residual	27	241.9		27	212.4	

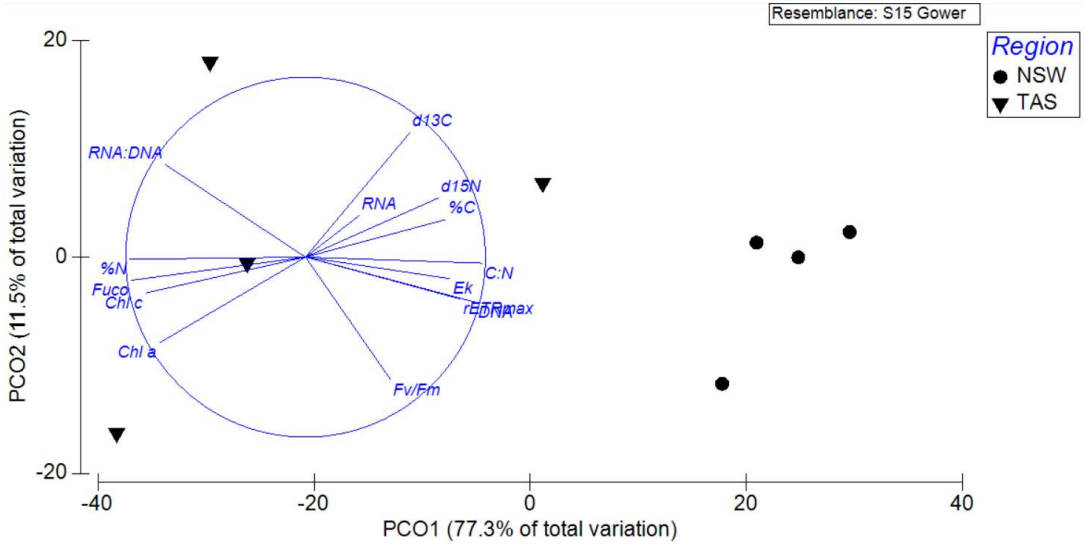

Figure 3.1 – Principle coordinates analysis (PCO) based on Gower's similarity matrix of untransformed multivariate physiology data from NSW and Tasmanian *Ecklonia radiata* sporelings in-situ.

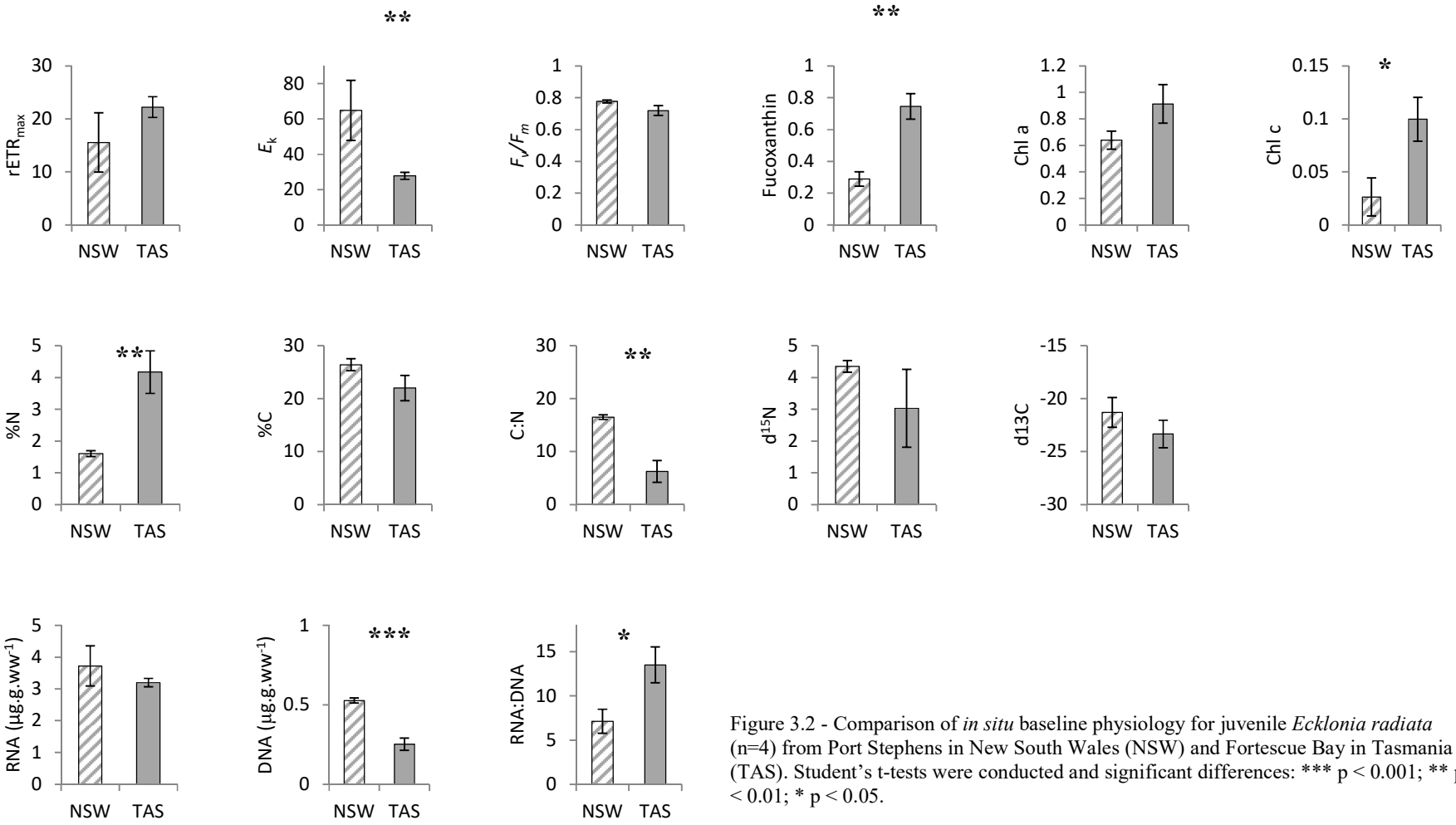

Figure 3.2 - Comparison of *in situ* baseline physiology for juvenile *Ecklonia radiata* (n=4) from Port Stephens in New South Wales (NSW) and Fortescue Bay in Tasmania (TAS). Student's t-tests were conducted and significant differences: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

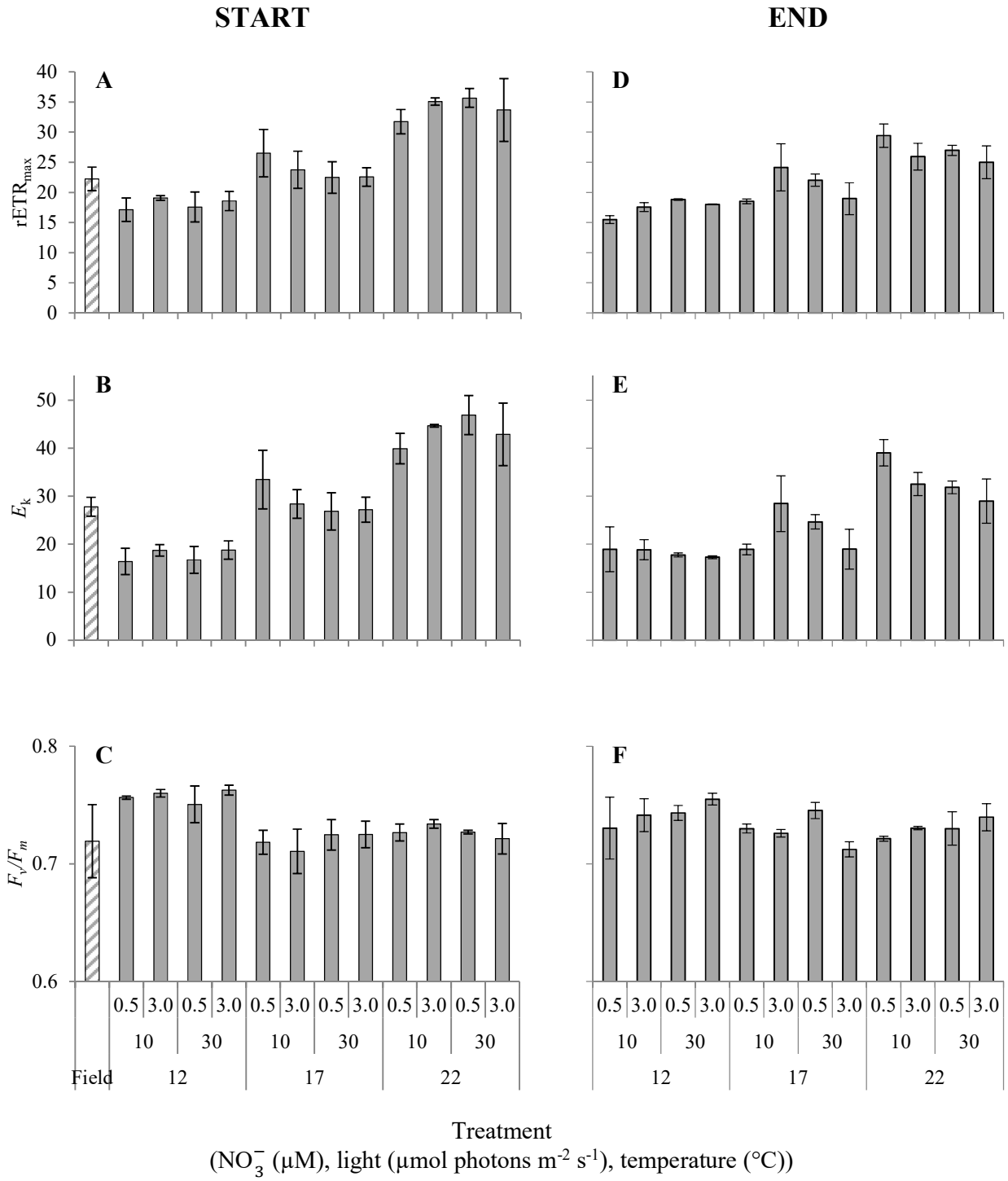

Figure 3.3 – Photosynthetic traits of *Ecklonia radiata* sporelings originating from Tasmania measured *in situ* (Field) and after growth in experimental treatments of temperature (3 levels), light (2 levels) and nitrate (2 levels) following acclimation to experimental conditions at T_0 (A-C) and T_{end} (D-F). Plots show (A, D) maximum relative electron transport rate ($rETR_{max}$); (B, E) saturating light intensity (E_k); (C, F) optimum quantum yield (F_v/F_m); as derived from dark-adapted RLCs measured by PAM fluorometry. Bars indicate mean values (n = 3) ± SE.

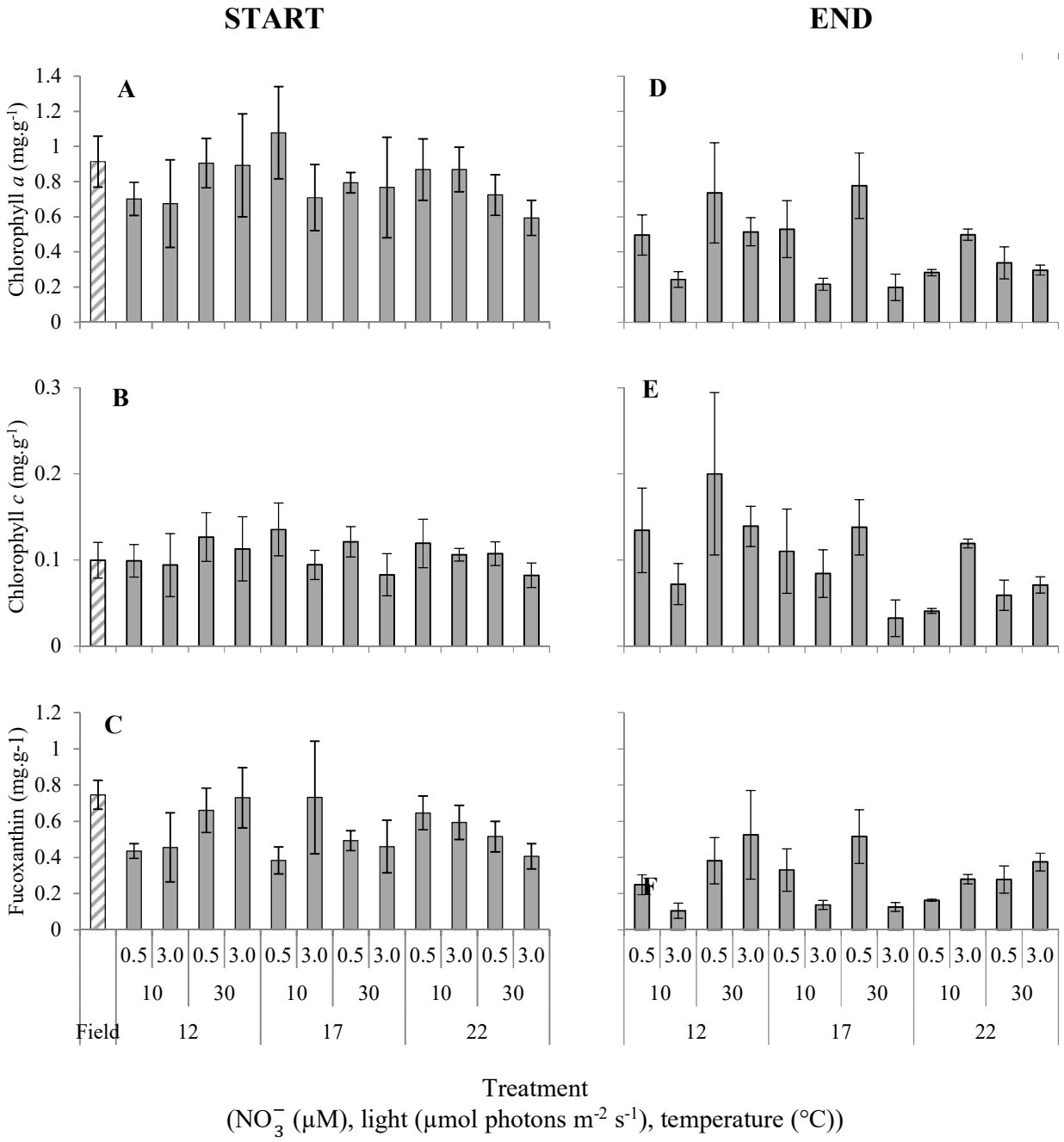

Figure 3.4 – Pigment content of *Ecklonia radiata* sporelings originating from Tasmania measured in situ (Field) and after growth in experimental treatments of temperature (3 levels), light (2 levels) and nitrate (2 levels) following acclimation to experimental conditions at T_0 (A-C) and T_{end} (D-F). Plots show tissue concentrations of (A, D) chlorophyll *a*; (B, E) chlorophyll *c*; (C, F) Fucoxanthin. Bars indicate mean values (n = 3) ± SE.

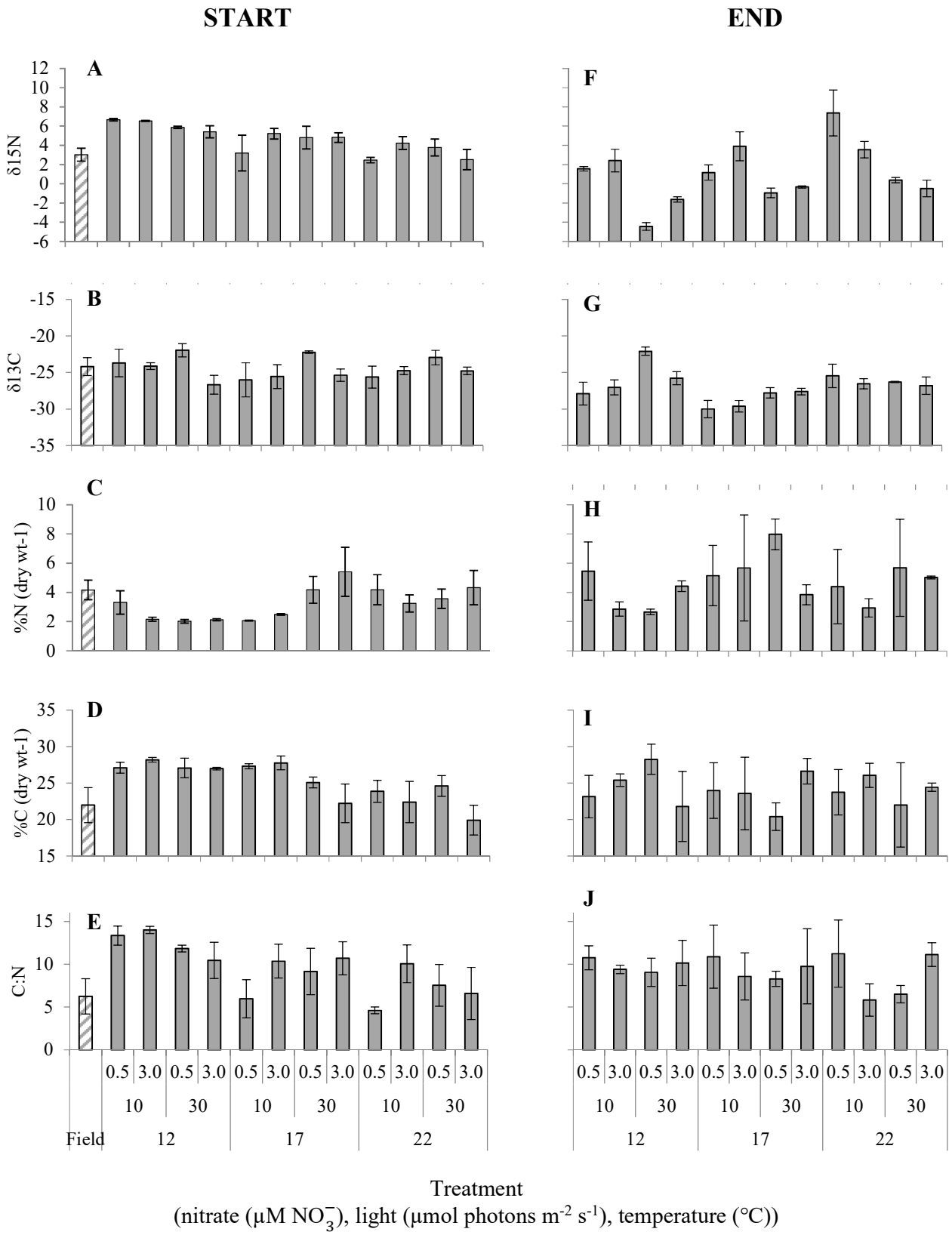

Figure 3.5 – Elemental content of *Ecklonia radiata* sporelings originating from Tasmania measured in situ (Field) and after growth in experimental treatments of temperature (3 levels), light (2 levels) and nitrate (2 levels) following acclimation to experimental conditions at T_0 (A-E) and T_{end} (F-J). Plots show isotope signatures for (A, F) nitrogen and; (B, G) carbon; (C, H); proportion of tissue comprising (C, H) nitrogen and; (D, I) carbon and; (E, J) carbon:nitrogen ratios. Bars indicate mean values (± SE).

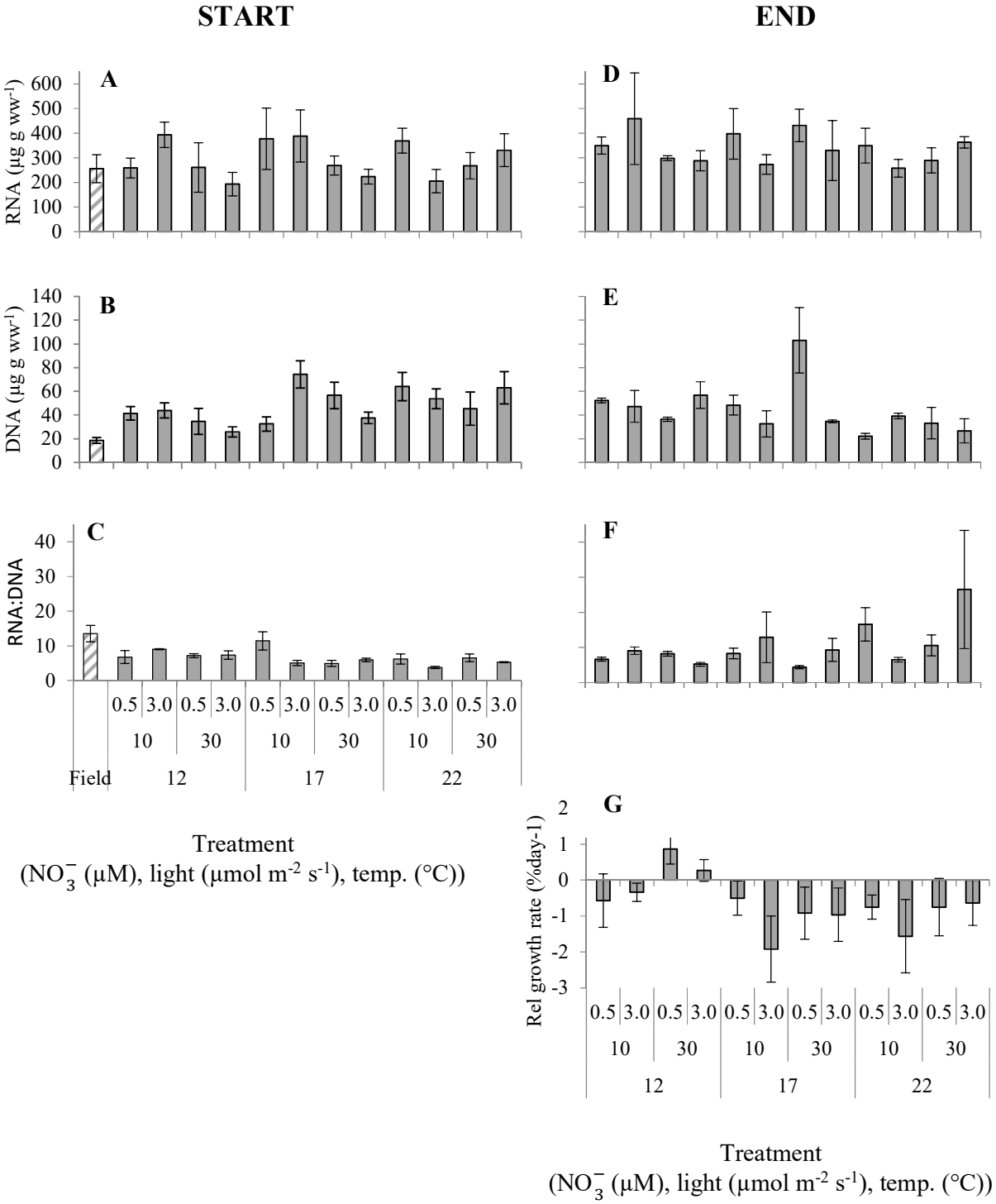

Figure 3.6 – Nucleic acid content and relative growth rate of *Ecklonia radiata* sporelings originating from Tasmania measured in situ (Field) and after growth in experimental treatments of temperature (3 levels), light (2 levels) and nitrate (2 levels) following acclimation to experimental conditions at T_0 (A-C) and T_{end} (D-G).). Plots show tissue concentrations of (A, D) RNA; (B, E) DNA; (C, F) RNA:DNA ratio and; (G) relative growth rate. Bars indicate mean values (± SE).

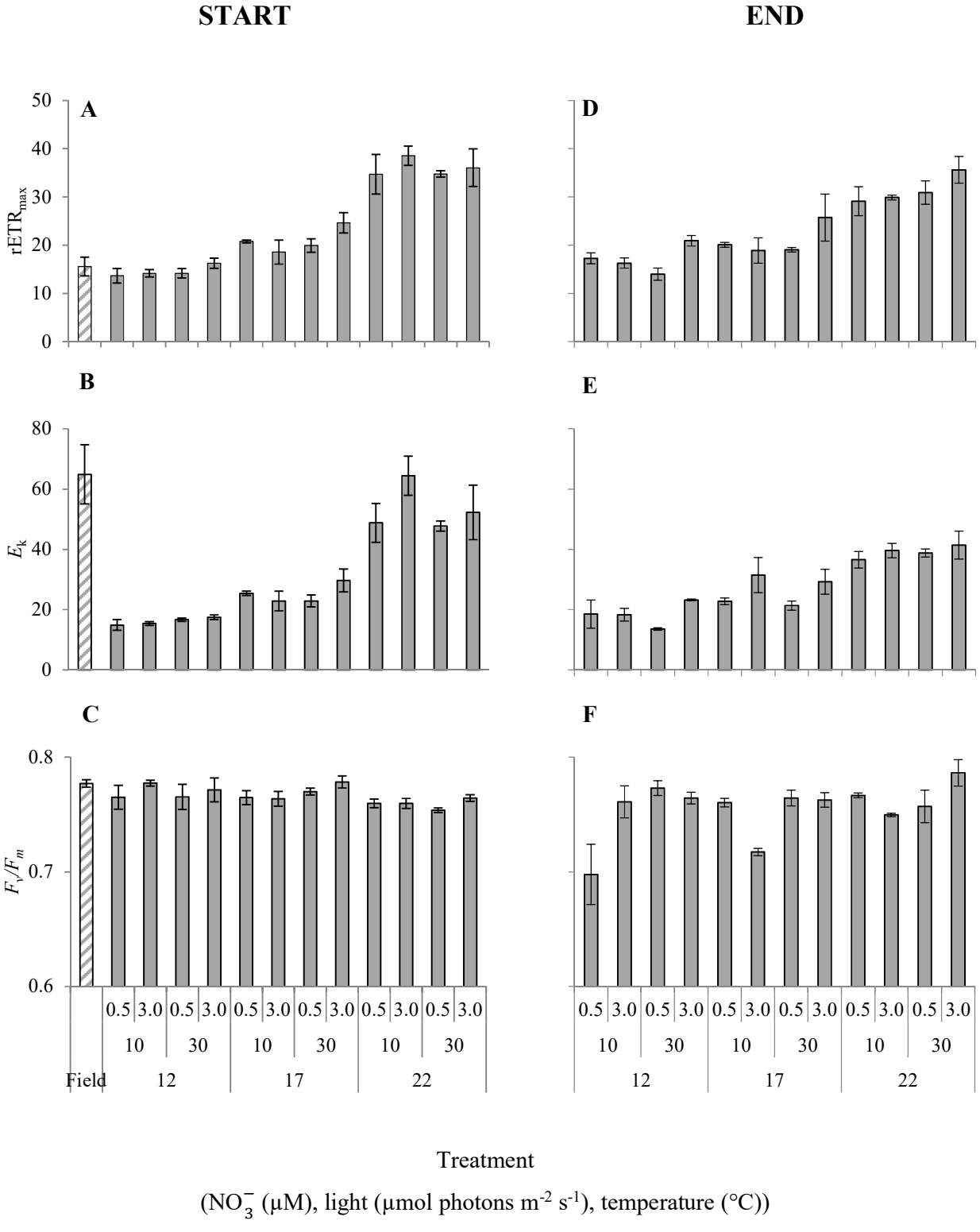

Figure 3.7 – PSII traits of *Ecklonia radiata* sporelings originating from NSW measured in situ (Field) and after growth in experimental treatments of temperature (3 levels), light (2 levels) and nitrate (2 levels) following acclimation to experimental conditions at T_0 (A-C) and T_{end} (D-F). Plots show (A, D) maximum relative electron transport rate ($rETR_{max}$); (B, E) saturating light intensity (E_k); (C, F) optimum quantum yield (F_v/F_m); as derived from dark-adapted RLCs measured by PAM fluorometry. Bars indicate mean values (n = 3) ± SE.

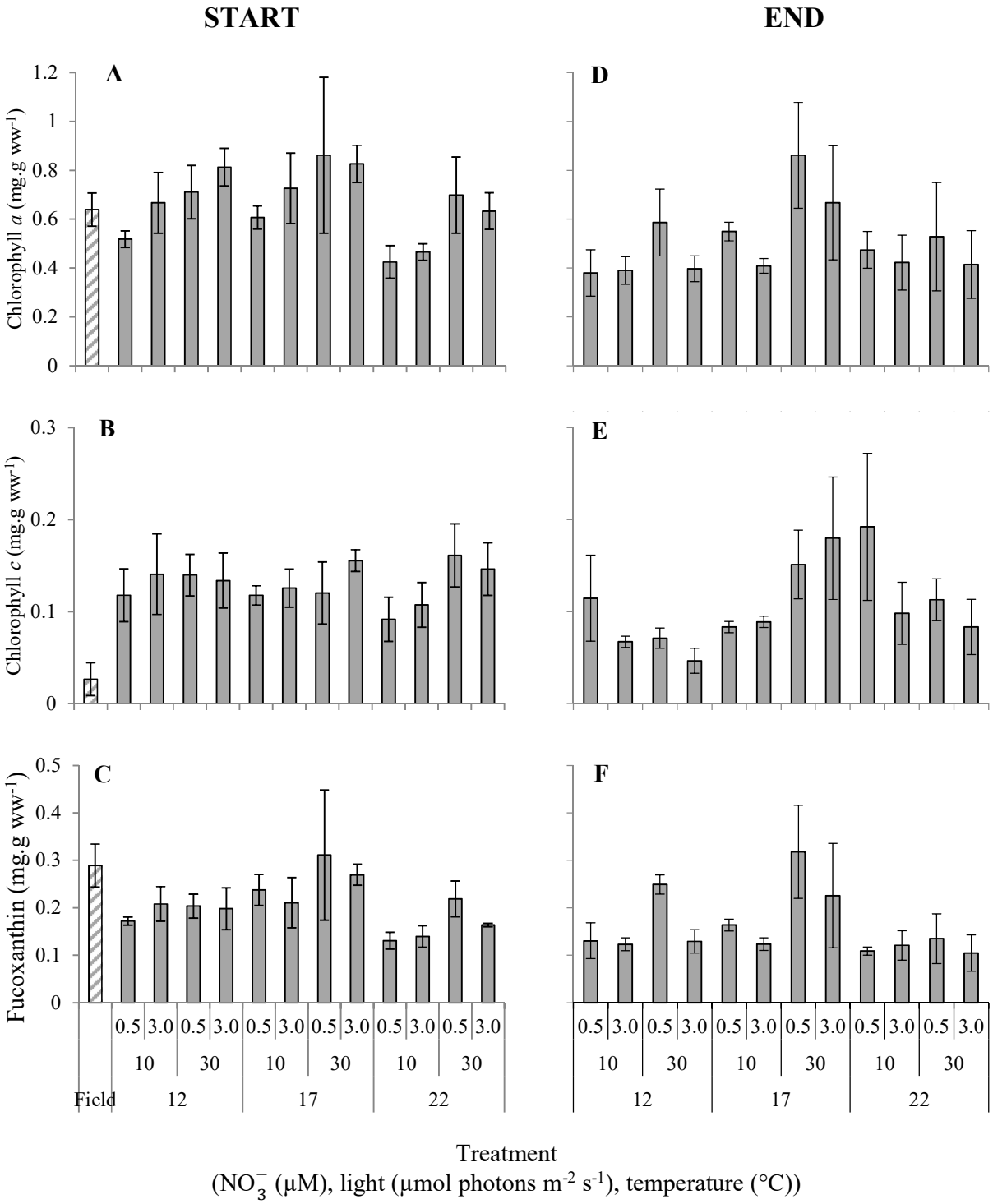

Figure 3.8 – Pigment content of *Ecklonia radiata* sporelings originating from NSW measured in situ (Field) and after growth in experimental treatments of temperature (3 levels), light (2 levels) and nitrate (2 levels) following acclimation to experimental conditions at T_0 (A-C) and T_{end} (D-F). Plots show tissue concentrations of (A, D) chlorophyll *a*; (B, E) chlorophyll *c*; (C, F) Fucoxanthin. Bars indicate mean values (n = 3) ± SE.

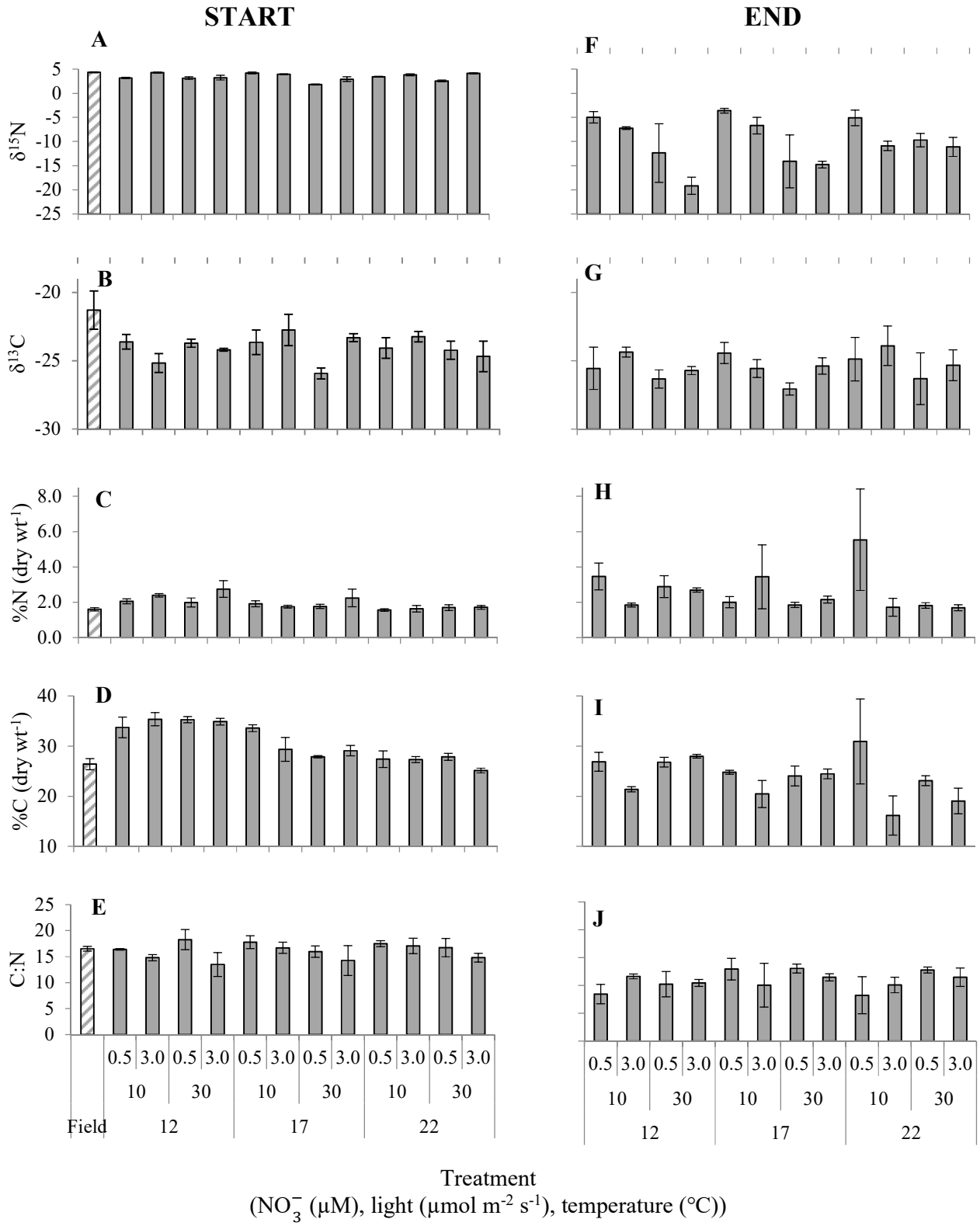

Figure 3.9 – Elemental content of *Ecklonia radiata* sporelings originating from NSW measured in situ (Field) and after growth in experimental treatments of temperature (3 levels), light (2 levels) and nitrate (2 levels) following acclimation to experimental conditions at T_0 (A-E) and T_{end} (F-J). Plots show isotope signatures for (A, F) nitrogen and; (B, G) carbon; (C, H); proportion of tissue comprising (C, H) nitrogen and; (D, I) carbon and; (E, J) Carbon:Nitrogen ratios. Bars indicate mean values (± SE).

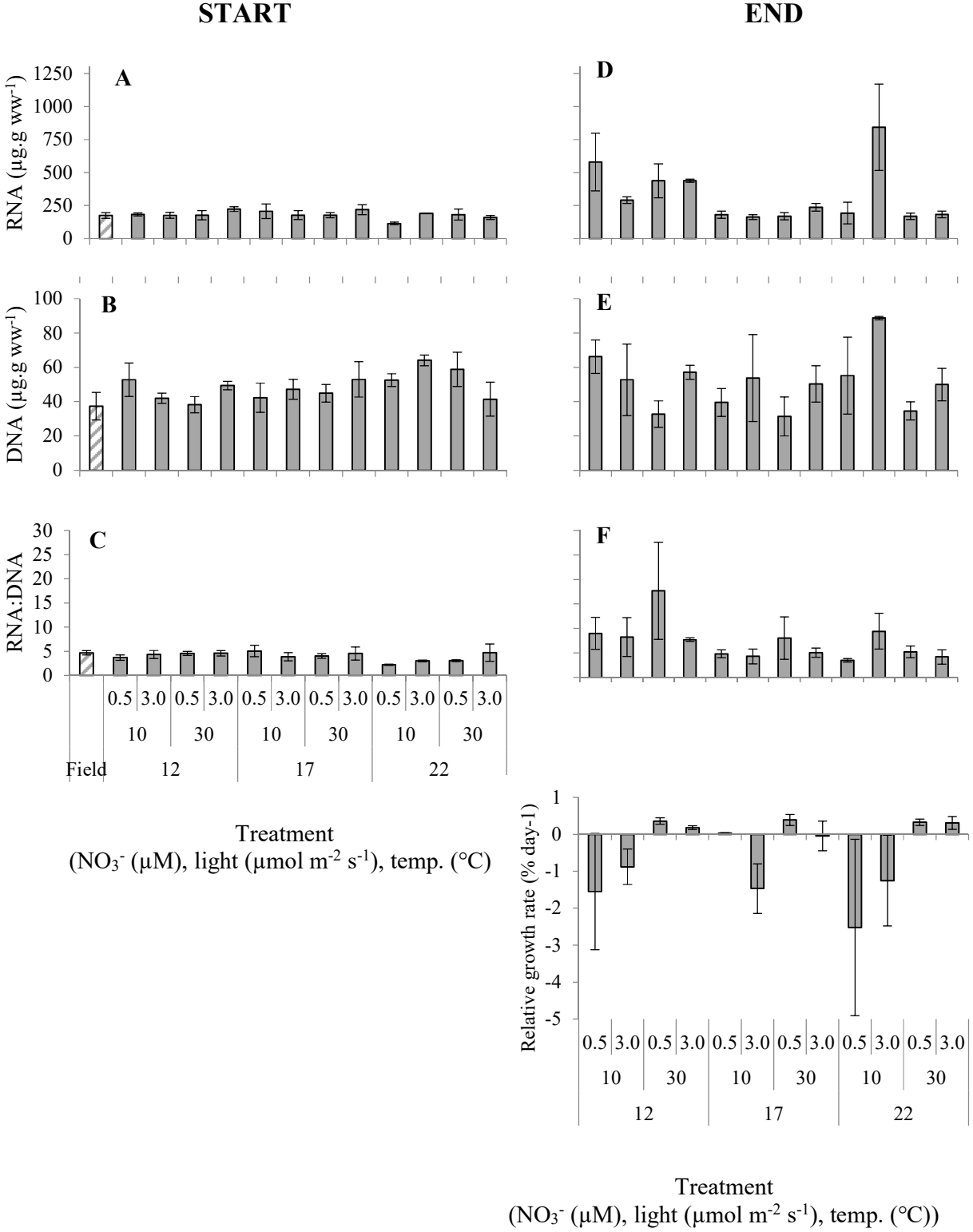

Figure 3.10 – Nucleic acid content and relative growth rate of *Ecklonia radiata* sporelings originating from NSW measured in situ (Field) and after growth in experimental treatments of temperature (3 levels), light (2 levels) and nitrate (2 levels) following acclimation to experimental conditions at T_0 (A-C) and T_{end} (D-G). Plots show tissue concentrations of (A, D) RNA; (B, E) DNA; (C, F) RNA:DNA ratio and; (G) relative growth rate. Bars indicate mean values (± SE).

3.5 Discussion

Our study revealed that temperature overwhelmingly drove variation in a range of different physiological variables of *Ecklonia radiata* sporelings sourced from NSW and Tasmania when grown at different temperature, light and nitrate levels. While temperature predominantly drove significant differences in physiology, seaweed from Tasmania and NSW responded differently to experimental treatments, and some main effects of light and nitrates, and higher-level interactions, emerged during the experiment.

3.5.1 Site differences in E. radiata *physiology (NSW v. Tasmania)*

Inherent differences between *E. radiata* sporelings in NSW and Tasmania measured *in situ* were evident at the time of collection. NSW sporelings were lower in accessory pigment concentration (fucoxanthin and chlorophyll *c*), nitrogen composition, and RNA:DNA ratios (driven by higher DNA concentrations), and showed a higher minimum saturating irradiance (E_k) relative to Tasmanian sporelings. All other physiological measurements ($rETR_{max}$, F_v/F_m, chlorophyll *a*, carbon concentration, isotopic N and C signatures and RNA concentration) were similar between sites and an F_v/F_m range of 0.7 – 0.8, was within the non-impaired range reported for brown seaweeds (Büchel & Wilhelm, 1993; Colombo-Pallotta *et al.*, 2006). Pigment concentrations are typically higher in environments with lower incident light (Wing *et al.*, 2007) and at the time of collection, Tasmania (in October) had lower monthly mean surface irradiance than NSW (in February) (4.3 kWh m^{-2} and 5.5 kWh m^{-2} Bureau of Meteorology 2016). Higher nitrate concentrations in the Tasmanian sporelings may be explained in-part by the seasonal timing of collection at the end of winter, when the influence of nutrient-rich Southern Ocean waters (Rochford, 1984) provides an opportunity for nutrient loading, a strategy yet to be confirmed in *E. radiata* but known in some Laminarian species (Gagné *et al.*, 1982; Stephens & Hepburn, 2016). In contrast, sporelings in NSW were

collected in March, towards the end of the growth season (Steinberg, 1995) when nitrogen stores may be depleted.

3.5.2 The effects of temperature on kelp physiology

Our study emphasises the important role of temperature in affecting the physiology of juvenile *E. radiata* from both northern (NSW) and southern (Tasmania) sites during both short-term acclimation and longer-term exposure. Enzyme catalysed reactions relating to PSII (RuBisCO and Calvin Cycle activity) are temperature dependent (Raven & Geider, 1988) and thermally labile (Wahid *et al.*, 2007), explaining why $rETR_{max}$ and E_k increase with temperature. This temperature-PSII relationship is well-known among autotrophs within their dynamic range, although seaweeds typically display a downregulation of photosynthetic activity in PSII (i.e. a drop in $rETR_{max}$ and E_k) and reduced F_v/F_m at sublethal temperatures (Heinrich *et al.*, 2012; Andersen *et al.*, 2013; Pereira *et al.*, 2015). Although our data show no evidence of down-regulation of PSII at high temperatures, sporelings from Tasmania displayed higher F_v/F_m (at T_0) and relative growth at 12 °C, and a high incidence of tissue necrosis at 22 °C, indicating optimum performance at lower temperatures. In contrast, whilst $rETR_{max}$ and E_k varied with temperature for NSW sporelings, there were no differences in F_v/F_m or relative growth between temperatures and a much lower incidence of tissue necrosis at high temperatures, indicating an absence of sublethal, systemic effects within the experimental temperature range.

Chemical reaction rates in kelp follow the Q_{10} concept, whereby photosynthetic rates, respiration and cellular processes typically increase with temperature (Hurd *et al.*, 2014b). Catalysed reactions (i.e. involving enzymes) respond to temperature less rapidly than uncatalysed reactions and may explain short-term temperature-derived differences in %C and %N, as C and N uptake involves numerous catalytic reactions and sporelings were in the process of maintaining cellular elemental ratios at T_0 (Giordano *et al.*, 2005).

Temperature tolerance varies considerably among the different stages of a photosynthetic organism's development (Wahid *et al.*, 2007), and at the latitudinal extremes of a species' distribution where a species may have distinctly different photosynthetic characteristics to conspecifics from the middle of its range (Flukes et al. 2015). Our findings reaffirm that the juvenile sporophyte stage is similarly susceptible to increased temperature, but less resilient than microscopic stages from the same site which show signs of sublethal effects or arrested development at > 22.5 °C (Mabin *et al.*, 2013). Thus, the early mortality and necrosis of seaweed tissue in Tasmanian *E. radiata* sporelings shows that elevated temperatures, particularly from heatwave events, may lead to greater mortality rates in seaweed populations from higher latitudes, similar to field observations of adult *E. radiata* dieback during heat-waves in Western Australia (Wernberg *et al.*, 2012a, 2016a). Whether our results for *E. radiata* sporelings also apply to fully developed adult sporophytes is unclear, as 'very' young sporelings of *Laminaria saccharina* are more susceptible to photo-damage and have a lower capacity to recover from environmental stress compared to older sporelings and adults (Hanelt *et al.*, 1997). In this context it can be noted that the large heatwave event in south-eastern Tasmania over the 2015-16 summer (~2.7 °C anomaly sustained for more than 130 days: Eric Oliver *pers. comm.*; Hobday et al. 2016) had little apparent impact on the morbidity and mortality of adult *E. radiata* sporophytes in the region (Masayuki Tatsumi *pers. comm.*). This event in Tasmania was greater in magnitude (in both duration and the extent of the anomaly) than the heatwave in Western Australia in the summer of 2010-11 which led to a ~100 km range contraction of kelp (Wernberg et al. 2016). This large-scale observation suggests that adult sporophytes in colder climes might be more resistant to a heatwave anomaly of given magnitude than their lower latitude conspecifics.

3.5.3 Other factors affecting kelp physiology

In contrast to the Tasmanian sporelings, F_v/F_m values and pigment concentrations in *E. radiata* sporelings from NSW showed no differentiation among any of the temperature, light or nitrate treatments (excepting a slight temperature effect for fucoxanthin in which levels at 17° C > 22° C).

In south-eastern Australia, the EAC influence is invariably < 3 µM nitrate and often less than 1 µM nitrate or undetectable (Harris *et al.*, 1987). The absence of nitrate effects on growth across the experiment was surprising given plentiful evidence linking nitrates to productivity (Pedersen & Borum 1996; Falkowski & Raven 1997; Reef et al 2012; Harrison & Hurd 2001). High nitrate treatments (3.0 µM nitrate) for Tasmanian sporelings had a higher optimum quantum yield (F_v/F_m) at 12 °C compared to 17°C and 22 °C, suggesting that cool-acclimatised *E. radiata* function more efficiently at the lower temperature when more ambient nitrate is available.

Light had surprisingly little effect on pigments and in some cases the effect was opposite to the expected inverse relationship between pigment concentration and high light intensity. Under low light, the fucoxanthin concentration of Tasmanian sporelings (at T_{end}) and the chlorophyll *a* concentration of NSW sporelings (at T_0) decreased. While it was expected chlorophyll *a* production to increase as a compensatory response to low light (Miller *et al.*, 2006), there is no logical explanation for the decline in chlorophyll *a* concentration in NSW sporelings at T_0, although by T_{end}, direction of the chlorophyll *a* response to light was as expected. Carotenoid accessory pigments such as fucoxanthin have other roles in addition to photosynthesis, functioning as an oxidization substrate, protecting cells and tissues against the harmful effects of high irradiance (Krinsky, 1978), which possibly explains reduced fucoxanthin concentrations under low light (Stengel & Dring, 1998; Yotsukura *et al.*, 2012). Reduced pigment concentrations were expected through loss of functionality for pigment

production at high temperatures and low nitrates (Hipkin *et al.*, 1983; Jokiel & Coles, 1990; Major & Davison, 1998; Staehr & Wernberg, 2009). Chlorophyll *c* in Tasmanian sporelings followed this prediction (possibly a synergistic effect of high temperature and low nitrates, Gerard 1997), however pigment concentration was higher in low nitrate conditions in Tasmanian sporelings at 17 °C and associated with increased utilisation of media N (lower $\delta^{15}N$ signatures). Rates of N turnover are primarily associated with temperature variation in Laminarians (Duke *et al.*, 1989) although N-utilisation dynamics (i.e. uptake, storage and assimilation) are poorly understood. Our data suggest uptake and assimilation systems were stimulated by N shortage as seen in microalgae and cyanobacteria (Beardall & Giordano, 2002) and associated with pigment production. N availability is thought to enable RubPCase activity in seaweeds (Küppers & Weidner, 1980) thus, low N availability reduces photosynthetic capability and pigments are produced to optimise photosynthetic capacity (Lapointe & Duke, 1984).

Nitrogen isotope signatures ($\delta^{15}N$) from sporelings in the experiment were more negative than those in the field at the time of initial collection (i.e. sporelings with higher tissue concentrations of media-derived nitrate yields more negative $\delta^{15}N$ values). Variation in nitrogen uptake rates in seaweed can be determined by factors such as nutritional history, age and surface area-volume ratio of the thallus (Harrison & Hurd, 2001). As sampling targeted the same area of the thallus on similar sized (and presumably similar aged) individuals, the large variation observed among individuals from the same site in Tasmania presumably reflects high phenotypic variability (for an unknown reason) despite close genetic similarity in *E. radiata* over vast distances (Coleman, 2013). Little is known of the process of N storage and utilisation in *E. radiata* although, other Laminarians exhibit seasonally-driven nitrogen utilisation dynamics strategies that are adapted to local conditions (Gagné *et al.*, 1982; Stephens & Hepburn, 2016).

Stable isotope fractions of $\delta^{13}C$ suggest Tasmanian seaweeds altered their carbon acquisition strategy to utilise more CO_2 under low light, whilst NSW showed no such response over the course of the experiment. Recent mechanisms of carbon acquisition can be inferred from $\delta^{13}C$ values. Seaweeds commonly utilise abundant bicarbonate (HCO_3^-) in photosynthesis with energy intensive carbon concentrating mechanisms (CCMs) and return $\delta^{13}C$ values between -10‰ and -29‰ (Raven *et al.*, 2002), while values more negative than -29‰ indicate exclusive uptake of dissolved CO_2 through passive diffusion on a concentration gradient (Raven, 2003; Raven & Hurd, 2012). Either these observations can be explained by adaptation of carbon use strategies typically seen in higher latitude seaweeds subject to low light environments (Hepburn *et al.*, 2011) to enable photosynthesis when light and nitrate is limiting (Beardall & Giordano, 2002) or the magnitude of difference between irradiance treatments was not sufficient to trigger a shift in carbon acquisition strategy in NSW sporelings. As CO_2 becomes more available in the oceans under climate change, some studies suggest this may work to oppose the negative impacts of increased temperature, as more carbon will be taken up passively, freeing up resources for protection against temperature increase. CO_2 also ameliorates negative effects of temperature stress on microscopic stages of *Macrocystis* (Roleda *et al.*, 2012; Gaitán-Espitia *et al.*, 2014).

3.5.4 Evidence for the GRH

The GRH presumes higher RNA to DNA ratios are linearly linked to growth rates. While cellular DNA concentration is constant, RNA is regulated for protein synthesis during growth processes (Dortch *et al.*, 1983; Elser *et al.*, 2003) thus it is predicted that organisms at higher latitudes, which have greater nutrient requirements (Elser et al. 2003), make them more susceptible to nutrient limitation. The GRH is supported in a range of fauna and bacteria species (Elser *et al.*, 2003; Acharya *et al.*, 2004) and, at least interspecifically (but not within species), among mangroves (Reef *et al.*, 2012), however, our data provides no evidence of a

link between relative growth rates and RNA:DNA ratios. At low temperature (12 °C), nutrient use corresponded well with the highest relative growth rates for Tasmanian *E. radiata* , while this was not evident for NSW *E. radiata*, lending partial support to the GRH prediction that higher latitude populations will make more efficient use of nutrients due to shortened growth seasons (Kerkhoff *et al.*, 2005; Lovelock *et al.*, 2007). However, the GRH has been inadequate at linking P, C:P ratios and RNA to growth for photosynthesising organisms, likely due to adaptations for coping with low or highly variable nutrient availability and the ability for substantial P storage in seaweed (Pedersen *et al.*, 2010). Furthermore, the complex protein requirements of seaweeds for biosynthesis processes are probable reasons for the incompatibility of applying the GRH to slow-growing seaweeds, hence intraspecific application of GRH linking relative growth rates and RNA:DNA ratios have not been found in seaweeds nor in other marine macrophytes (e.g. Reef et al. 2010, 2012; Flukes unpub data).

3.5.5 The efficacy of a multifactor approach

This study reiterates the importance of temperature in limiting kelp distribution (Marzinelli *et al.*, 2015) by affecting the physiology of the sporeling recruit stage of *E. radiata* (Wernberg et al. 2012b, Mohring et al. 2014), and demonstrates that light and nitrate are important in mediating these effects. This study emphasises the importance of latitudinal comparisons when studying the impacts of climate change on an important and widely distributed foundation species. The differences between Tasmania and NSW sporelings may be reflective of latitudinal differences but this conclusion needs to be made cautiously as region was not replicated due to the large number of physiological metrics measured in this study.

When studying physiological response of organisms to climate change, the investigator is limited to making inferences about physiological impacts relating only to the data collected, or provide proof of proxy measurements, in the case of the GRH, supported by known

physiological pathways. However, in autotrophs, these links are not well understood and the GRH exemplifies the dangers of describing complex biological systems using simple models with limited explanatory elements. This study highlights the complexities in determining which measures are effective predictors for future condition of populations under climate change, particularly for species with a high ecological value such as *E. radiata*. The apparent complexity of relationships among different physiological pathways in *E. radiata* (i.e. photophysiology, nutrient dynamics and growth) reaffirms that assessing physiological performance using fewer metrics restricts the interpretation of the overall physiological response of an organism to climate change. Furthermore, the effects of single stressors provides limited information of physiological response to climate change, and as demonstrated here, there is a strong justification for multifactor approach as evidenced by the frequent finding of interactive effects. In this context, experiments dealing with multiple stressors rarely capture natural dynamic fluctuations where factors are held constant throughout experiments (like this experiment). Experiments that mimic natural fluctuations are known to provide further insight into physiological response (i.e. Britton et al. 2016) as they capture the dynamic nature of abiotic stressors and should be incorporated into future climate change research (Gunderson *et al.*, 2016).

Chapter 4 | Family-level variation in early life-cycle traits of kelp: adaptive potential to climate change?

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4.1 Abstract

Temperate kelp forests (Laminarians) are threatened by temperature stress due to ocean warming and photoinhibition due to increased light associated with canopy loss. However, the potential for adaptation in kelp to rapid climate change is not well known. This study examined family-level variation in physiological and photosynthetic traits in the early life-cycle stages of the ecologically important Australasian kelp *Ecklonia radiata* and the response of *E. radiata* families to different temperature and light environments using a family x environment design. There was strong family-level variation in traits relating to size, fitness and performance in both haploid (gametophyte) and diploid (sporophyte) stages of the life-cycle. Additionally, the presence of family x environment interactions showed that offspring from different families respond differently to temperature and light in several gametophyte traits, with an overall negative response to high temperatures and stronger effects for females v. males. Our findings suggest *E. radiata* may be able to respond adaptively to climate change but studies partitioning the narrow vs. broad sense components of heritable variation are needed to establish the evolutionary potential of *E. radiata* to adapt under climate change.

4.2 Introduction

Climate change is modifying environmental conditions across the earth, placing ecosystems and the species they support under threat (IPCC 2014). Species can respond to these changes

by undergoing range shifts (Burrows *et al.*, 2011) or by adjusting their phenology, behaviour, physiology or morphology (e.g. Bradley et al. 1999, Menzel & Fabian 1999, Pulido et al. 2001, Li & Denny 2004, McGaugh et al. 2010). Many life-history traits show phenotypic plasticity allowing a response within an individual's life-time (Nylin & Gotthard, 1998; Hoffmann & Merilä, 1999) and much work has focussed on understanding plastic responses to novel stressors (see Padilla & Savedo 2013). However, because climate change creates new selection regimes, evolutionary adaptation of species is critical in their long-term response (Hoffmann & Sgro, 2011; Kelly & Hofmann, 2013; Reusch & Boyd, 2013). Despite its importance, most research of the response of marine species to climate change does not consider the evolutionary potential for adaptation (Munday *et al.*, 2013), limiting the capacity to predict how marine species will adjust to climate change.

The evolutionary potential to climate change will depend on heritable genetic variation for key traits (Lynch & Walsh 1998, Hoffman & Sgro 2011). In nature, morphological and phenological traits typically have significant levels of additive genetic variance with average narrow-sense heritabilities of 0.46 for morphological traits, and 0.26 for life history traits (Mousseau & Roff, 1987; Hendry, 2013). In terrestrial plants, ecologically important traits such as leaf number, leaf thickness and development rate show heritable variation (Jump & Penuelas, 2005) and can result in adaptive responses to climate stress (Agrawal *et al.*, 2008). Similar studies demonstrating evolutionary potential in marine species to adapt to climate change are less common (see review by Reusch 2014), however are increasing for marine invertebrates (see review Foo & Byrne, 2016). Many of these tests rely on estimates of broad-sense heritability (Császár *et al.*, 2010) which provides an upper estimate of heritability due to potential inheritance of non-genetic components. In addition to understanding trait heritability, the relative performance of genotypes across multiple environments (Bowman, 1972) (via genotype x environment studies) is a means of exploring the adaptive response of

quantitative traits (e.g. describing phenology, morphology and physiology: Galletly et al. 2007, Hawkins et al. 2010, Pease et al. 2010, McKenzie et al. 2012) to environmental stressors.

Habitat-forming seaweeds are ecologically important and form the foundation of many temperate reef ecosystems around the globe (Bolton, 1996; Beardall *et al.*, 1998; Kerswell, 2006; Steneck & Johnson, 2013). Kelps (order Laminariales) are sensitive to changes in temperature and light (Yarish *et al.*, 1990; Steneck *et al.*, 2002), making them susceptible to rapid climate change. Above average temperatures can cause reduced performance in kelp leading to kelp canopy loss (Wernberg *et al.*, 2010; Johnson *et al.*, 2011; Andersen *et al.*, 2013) and increased irradiance to the benthos which can result in a higher cover of understory species which limits kelp recruitment (Kennelly, 1987; Connell & Russell, 2010b; Tatsumi & Wright, 2016). Seaweeds are known to have a plastic response in morphology and physiology to changes in temperature (Hurd *et al.*, 2014c; Reusch, 2014; Flukes *et al.*, 2015), wave exposure (Blanchette, 1997; Fowler-Walker *et al.*, 2006), and light (Monro & Poore, 2005) but little is known of their potential for evolutionary responses. Heritable variation exists for chemical defences and growth in the brown alga *Fucus vesiculosis* and the red alga *Delisea pulchra* (Jormalainen & Honkanen, 2004; Wright *et al.*, 2004a), while the growth and photosystem traits of the brown alga *Hormosira banksii* show a (male) family x temperature interaction (Clark *et al.*, 2013). Because climate change is characterised by changes in multiple factors concurrently, understanding the response of species to multiple stressors is important in understanding adaptive capacity in habitat-forming seaweeds.

The kelp *Ecklonia radiata* (C. Agardh) (hereafter *Ecklonia*), is the most abundant and widespread habitat-forming macroalga in southern Australia (Womersley, 1981). Like other kelp, *Ecklonia* performance (growth, reproduction, mortality) is strongly influenced by temperature (latitudinal) and light (depth and kelp canopy cover). In Tasmania, south eastern

Australia, ocean temperatures are warming at a rate of almost four times the global average due to intensifying southerly incursions of warm, nutrient poor waters of the east Australian current (EAC) (Ridgway, 2007b). Negative effects of elevated temperatures on *Ecklonia* at multiple life stages can lead to a thinning of the adult *Ecklonia* canopy (Wernberg et al. 2010), range contraction (Wernberg *et al.*, 2012a) and regime shifts (Wernberg *et al.*, 2016a). Early life stages of *Ecklonia* are negatively impacted by elevated temperatures but not nitrates (Mabin *et al.*, 2013; Mohring *et al.*, 2014) while high light that occurs following canopy reduction reduces gametophyte recruitment and increases microscopic sporophyte recruitment but only when zoospore density is high (Tatsumi & Wright, 2016). Additionally, high temperature and high light have additive negative effects on larger macroscopic recruits, likely due to photoinhibition (Mabin et al. in review). Thus although canopy thinning in the field often results in an increase in juvenile sporophyte abundance (Kirkman 1981, Kennelly 1987, Flukes et al. 2014) the response of microscopic and juvenile sporophytes to the increased light appears complex and likely to interact with other environmental stressors. There is high phenotypic plasticity in *Ecklonia* to environmental factors (Wernberg & Thomsen 2005, Fowler-Walker et al. 2006), but the possible adaptive responses are not known.

This study determined the family-level variation in traits of the early life-cycle stages of *Ecklonia* to new selective regimes predicted under climate change. Because selection can impact life-stages differently (Harvey *et al.*, 2014), responses were examined across two stages of development. First, family-level variation was determined for multiple quantitative traits in haploid (male and female gametophytes) and diploid (sporophyte) life-cycle stages of *Ecklonia* (experiments 1 & 2). Because *Ecklonia* sporophytes are monoecious and release male and female zoospores simultaneously it was not possible to partition sire and dam effects and determine narrow sense heritability and additive genetic variance for traits. Thus, our

estimates represent the upper bounds (broad-sense) heritability in these traits and are an important first step in understanding the potential for adaptive responses in *Ecklonia*. It was then determined whether families (gametophytes) respond differently to crossed temperature and light treatments that reflected current and predicted conditions using a genotype x environment design (experiment 3).

4.3 Materials and methods

4.3.1 Ecklonia radiata *reproductive biology*

Ecklonia radiata is a perennial subtidal kelp that grows to a maximum of 2 meters in height (Womersley 1987). Reproduction is seasonal, with a maximum zoospore production in eastern Tasmania during cooler months (Sanderson, 1990; Mabin *et al.*, 2013), peaking around May (late autumn). Sporangia develop below the surface forming sorus tissue on lateral fronds and the distal end of the central lamina. Zoospores are produced via meiosis within sporangia then released from the sori onto the thallus surface. Swimming competency of *Ecklonia* zoospores is not known but up to 72 hours has been recorded in other kelp (Reed *et al.*, 1992). Zoospores then settle to the benthos where gametogenesis occurs. Gametophytes are dioecious and filamentous, exhibiting sexual dimorphism. Females reach up to 400 µm in length while males reach up to 100 µm in length. Female gametophytes produce non-motile reproductive cells (the oogonia), which are fertilised by mobile antherozoids released from antheridia cells in male gametophytes. Zygotes develop into sporophytes, consisting of a single frond growing from a stipe, and a holdfast for attachment to the substratum. In Tasmania, the early *Ecklonia* sporophyte stage requires temperatures below 22.5°C and low irradiance to persist and grow (Mabin *et al.*, 2013; Mohring *et al.*, 2013).

4.3.2 Collection of reproductive tissue

Sori were collected at 10-12 m depth from Fortescue Bay, Tasmania (43° 7’ 23.32” S, 147° 58’ 34.37” E) on two occasions: June 2012 (experiment 1: family effects) and October 2013

(experiment 2: family effects on F_v/F_m and; experiment 3: family x environment experiment; see below). To minimise damage to the kelp bed, sori were sliced from reproductive *Ecklonia* adult thalli using a knife. Single pieces of sori were collected from 20 (family experiment 1), 16 (family experiment 2), and 4 (experiment 3) sporophytes and transported in seawater to the IMAS aquaculture facility in Launceston, Tasmania.

4.3.3 Culturing protocol

Zoospore liberation followed methods described in Mabin et al. (2013), with zoospores from each family released into both separate stock containers and a mixed stock container consisting of zoospores from all families. Wright's Chu culture media (WC media) comprising UV treated, 0.2 µm filtered seawater base, with nutrients, trace metals and vitamins (Andersen, 2005) were used during sporulation and culturing.

Zoospores were added to 50 ml plastic jars (n = 6 per family) containing media to achieve a concentration of ~2,000 zoospores ml^{-1}. Four round coverslips (13 mm diameter) placed on the bottom internal surface of the jar provided a substratum for zoospore settlement and development, enabling destructive sampling of gametophytes and sporophytes at different times. Families and the mixed treatment were randomly arranged under lights.

Culturing was done at 15 °C and 30 µmol photons $m^{-2}s^{-1}$ irradiance (except in the G x E experiment where there were two levels each of the temperature and light treatments; see below), which reflected average Fortescue Bay spring temperatures and understory irradiance in October (Tatsumi, unpub. data). Lighting consisted of parallel 40 watt Sylvania standard cool white globes (model F40W/133-RS) on a 12:12 light-dark cycle with incidental irradiance set to ~30 µmol photons m^{-2} s^{-1}. Every seven days ~ 90% of the media was gently refreshed using a syringe to reduce disturbance to the gametophytes and sporophytes in the cultures.

4.3.4 Experimental design: family effect experiments (experiments 1 and 2)

Two experiments determined family-level variation in traits of male and female gametophytes and sporophytes of *Ecklonia*. In the first experiment, 20 families (N = 6 per family) and a mixed treatment (i.e. zoospores from all 20 families mixed together) were cultured and morphological traits measured on gametophytes and sporophytes as described below. In the second experiment, 16 families (N = 5 per family) and a mixed treatment were cultured and maximum quantum yield (F_v/F_m) of sporophytes was measured as described below.

4.3.5 Experimental design: family x environment experiment (experiment 3)

To test whether families responded differently to different environmental conditions, four families (random factor) were cultured under two levels of temperature (16 and 22 °C, fixed) and irradiance (10 and 30 µmol photons $m^{-2}s^{-1}$, fixed: N = 3 per treatment combination). Conditions were chosen to mimic future temperature conditions in Tasmania (Ridgway & Hill, 2012) and light-levels reflect high to low canopy densities (Tatsumi & Wright, 2016) as it is predicted that kelp destabilisation under climate change will lead to reduced canopy cover (increased light). To create the low light treatment, jars were covered with layers of flyscreen mesh. Irradiance was measured inside the jars using a LI-COR 1400 light meter to verify treatment levels. This experiment only determined the response of gametophytes as no sporophytes developed at 22 °C. After 14 days the same morphological traits were measured on gametophytes as in the family-level experiment. In addition, maximum quantum yield (F_v/F_m) of the female oogonia cells and male vegetative (apical) cells was determined.

4.3.6 Trait measurement

Traits that were considered potentially relevant as responses to climate change were growth, development, reproduction, and photosynthesis. Measurements were taken from one haphazardly selected gametophyte (after 14 days) and sporophyte (at 28 days) from a haphazardly selected coverslip within each jar.

Morphological traits were measured using image analysis (ImageJ v1.48) of photographs taken of haphazardly selected frames of coverslips with a Canon EOS 20D and microscope eyepiece attachment attached to a compound microscope at 400x magnification for the gametophyte stage and 100x magnification for microscopic sporophytes. Up to ten photographs were taken haphazardly of each coverslip but only a single photograph per coverslip was chosen randomly for image analysis. A 10 x 10 grid was placed on the chosen image and coordinates were randomly generated, and measurements were taken from the closest specimen. Measurement scales were calibrated at each magnification scale using a digital image of a calibration slide (Wild Heerbrugg Switzerland Model 310345). For male and female gametophytes total surface area, maximum branch length and branch count were measured, while a count of total cells and the surface area of oogonia were also measured on females. Individual cells were difficult to distinguish in male gametophytes and were not measured. Total surface area, thallus length, and thallus width were measured on sporophytes.

Chlorophyll fluorescence of gametophytes and sporophytes was measured using a red light pulse amplitude modulated (PAM) fluorometer, consisting of a photomultiplier, customised and mounted onto a microscope (Microscope-PAM Walz, Effeltrich, Germany). Maximum quantum yield or intrinsic potential efficiency of PSII (F_v/F_m) was determined from dark-acclimated samples where minimum (F_0) and maximum (F_m) fluorescence were used to calculate variable fluorescence (F_v) prior to calculating the maximum quantum yield of PSII (F_v/F_m). Replicate coverslips were removed from treatment jars, loaded onto microscope slides and dark-acclimated for 15 minutes (McMinn *et al.*, 2005). Slides were placed on the microscope stage with a black cloth draped around the microscope PAM where readings were taken. Fluorescence was measured in the centre of an oogonia (female) or apical vegetative (male) cell on randomly selected gametophytes.

4.3.7 Statistical analyses

Analysis of variance (ANOVA) was used to determine significant differences between families for traits, and Pearson's correlations between traits were performed on family means for traits to assess potential for intra- and inter-generational correlations. ANOVA and PERMANOVA (Anderson *et al.*, 2008) were performed on univariate and multivariate traits to test for three-way interactive effects of family, temperature and light. For all ANOVAs, the Box-Cox procedure was applied to determine the appropriate transformation (if any) to stabilise variances . PERMANOVA used Bray-Curtis similarity matrices determined from square-root transformed data to estimate the significance of treatment effects on the joint distribution of all traits. Where the number of unique values in the permutation distribution was less than 999, Monte Carlo *P*-values were used instead of permutational *P*-values (Anderson *et al.*, 2008). Significant effects were examined using post-hoc pairwise tests. A backwards-selected linear mixed model, created with nlme (Pinheiro *et al.*, 2014) in 'R' (v 3.0.0), was used to estimate bivariate correlations and covariance matrices between all traits of males, females and sporophytes. Data were scaled and centred prior to analysis and a meta-analytic approach was used to account for non-independence and error associated with the estimates of family means. All univariate, linear mixed model and correlation analyses were performed using the package 'R' (v 3.0.0), and PERMANOVA analyses were generated using PRIMER-E software (v 6.1.12) with the PERMANOVA+ add-on package.

4.4 Results

4.4.1 Family-level variation in key physiological traits

Traits of *Ecklonia* gametophytes and sporophytes varied significantly among families with the exception of branch length of male gametophytes (Table 1; Fig. 1). For the gametophytes, morphological traits varied among families by up to 3-4 times, while for sporophytes most traits varied by an order of magnitude among families. Sporophyte F_v/F_m also showed

significant family-level variation (Fig. 2). All correlations within and between gametophyte-sporophyte stages were positive and significant although there were weaker relationships (smaller Pearson *r*) between life-cycle stages (Fig. 3). Mixed treatments trait values were generally close to the family average for all traits (Figs. 1 & 2).

4.4.2 Trait correlations and variance-covariance estimates

Multivariate linear mixed model indicated significant overall bivariate correlations and covariance estimates between all eleven morphological traits in *Ecklonia radiata* gametophytes (male and female) and sporophytes traits. Phenotypic correlations were all positive, with relative strength of correlations high among males and female gametophyte traits and low between sporophyte and gametophyte traits (Table 4.4).

4.4.3 Effects of family, temperature and light

There was a significant interaction between family and irradiance for oogonia surface area of female gametophytes, indicating genetic variation in light sensitivity for this trait (Fig. 4; Table 2). There was also a significant interaction between family and temperature for the number of branches of male gametophytes, indicating genetic variation in thermal sensitivity (Fig. 4; Table 2). The surface area of male and female gametophytes, branch length of female gametophytes, and number of branches of male gametophytes were all significantly greater when grown in the higher light treatment, while female gametophyte surface area, branch number, and cell number were significantly greater at the higher temperature (Fig. 4; Table 2). In addition, several traits also differed significantly among families (Fig. 4; Table 2). No significant effects of family, temperature, or light were detected for branch length (male gametophytes) or maximum quantum yield in gametophytes for which absolute values ranged 0.004 – 0.665 (males) and 0.015 - 0.716 (females) (Fig. 4; Table 2).

PERMANOVA identified significant differences in the multivariate morphology of male and female gametophytes among families, and across light levels (Table 3). Female gametophyte multivariate morphology also varied significantly between temperatures. These differences were supported visually by clear patterns of family and light (males & females) and temperature (females) on CAP plots (Fig. 5). However, there were no significant family x environment interactions for either male or female gametophytes.

Table 4.1 – Summary table of ANOVAs testing the effects of family (random effect) on traits of male and female gametophytes and sporophytes of *Ecklonia radiata*. n = 20 for all traits except for sporophyte F_v/F_m where n = 16. Significant tests shown in bold. SA_{TOT} – total surface area; BL_{max} – maximum branch length; n branch – number of branches; $SA_{Oogonia}$ – Oogonia surface area; n cell – number of cells; TL – thallus length; TW – thallus width.

	Female gametophytes										
Source		**SA_{TOT}**		**BL_{max}**		**n branch**		**$SA_{Ooginia}$**		**n cell**	
	df	**MS**	**F**	**MS**	**F**	**MS**	**F**	**MS**	**F**	**MS**	**F**
Family	19	0.006	**12.1*****	0.512	**6.81*****	0.752	**6.70*****	0.161	**3.22*****	0.063	**5.13*****
Error	100	0.001		0.075		0.112		0.050		0.012	
	Male gametophytes										
		SA_{TOT}		**BL_{max}**		**n branch**					
	df	**MS**	**F**	**MS**	**F**	**MS**	**F**				
Family	19	0.011	**12.3*****	3.265	1.58	2.310	**10.7*****				
Error	99	0.001		2.073		0.215					
	Sporophytes										
		SA_{TOT}		**TL**		**TW**		**F_v/F_m**$^{\#}$			
	df	**MS**	**F**	**MS**	**F**	**MS**	**F**	**df**	**MS**	**F**	
Family	18	0.201	**10.6*****	3.480	**8.75*****	0.070	**10.9*****	19	0.092	**9.61*****	
Error	88	0.019		0.398		0.006		91	0.010		

Tests of significance: *** P < 0.001

Table 4.2 – Summary table of three-way mixed-model ANOVA testing the effects of Family (4 levels: random); temperature (2 levels: fixed); and light (2 levels: fixed) on traits of female and male gametophytes of *Ecklonia radiata*. F-ratios are shown with significant tests in bold. SA_{TOT} – total surface area; $SA_{Oogonia}$ – Oogonia surface area; BL_{max} – maximum branch length; n branch – number of branches; n cell – number of cells; F_v/F_m – maximum quantum yield.

	Female Gametophyte							Male gametophyte			
Factor	***df***	**SA_{TOT}**	**$SA_{Oogonia}$**	**BL_{max}**	**n branch**	**n cell**	***F_v/F_m***	**SA_{TOT}**	**BL_{max}**	**n branch**	***F_v/F_m***
Family (F)	3,0.213	**9.869*****	2.793	**3.564***	2.745	**3.806***	0.441	**4.043***	0.791	**11.96*****	0.149
Temperature (T)	1,3	**13.96*****	1.402	0.093	**9.079****	**12.06****	0.416	4.190	1.071	0.050	0.001
Light (L)	1,3	**37.50****	4.320	**13.14***	0.899	**6.497***	0.740	**48.55***	9.960	**60.54****	0.122
F x T	3,3	2.430	0.281	1.133	2.129	1.412	0.349	1.261	1.085	**3.195***	0.168
F x L	3,3	0.680	**4.481****	0.667	0.682	0.687	0.786	1.540	1.728	1.581	0.219
T x L	1,3	2.015	0.233	3.018	0.971	1.607	0.965	0.901	0.697	0.875	2.048
F x T x L	3,32	2.509	2.227	1.781	1.041	1.344	0.319	0.279	2.215	1.620	0.240

Tests of significance: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 4.3 – Permutational multivariate analysis of variance (PERMANOVA) testing the effects of family (4 levels: random), temperature (2 levels: fixed) and light (2 levels: fixed) on multivariate morphology of *Ecklonia radiata* gametophytes. P-values in bold indicate statistical significance ($p < 0.05$) based on 9999 permutations except for tests for the effects of light and temperature where 425 permutations were performed.

	Female gametophytes				**Male gametophytes**			
Source	**df**	**MS**	**Pseudo-F**	**P(perm)**	**df**	**MS**	**Pseudo-F**	**P(perm)**
Family (F)	3	216.7	5.1294	**0.001**	3	215.58	5.0522	**0.002**
Temperature(T)	1	385.3	7.1041	**0.037**	1	122.05	1.9691	0.235
Light (L)	1	712.3	13.367	**0.011**	1	1875.6	30.071	**0.004**
F x T	3	54.24	1.2837	0.279	3	61.984	1.4526	0.226
F x L	3	53.29	1.2612	0.277	3	62.37	1.4617	0.225
T x L	1	133.2	1.8349	0.244	1	31.542	0.7162	0.567
F x T x L	3	72.60	1.7183	0.142	3	44.04	1.0321	0.397
Residual	32	42.25			32	42.67		

Table 4.4 – Phenotypic correlations (above the diagonal), phenotypic covariance (below the diagonal) and trait variance (diagonal – shaded) among eleven morphological traits in *Ecklonia radiata* gametophytes (female and male) and sporophytes, estimated from family-level (random) linear mixed model measured from 20 families (n = 6 replicates).

		Female					Male			Sporophyte		
	Traits	branch count	cell count	branch length	Oogonia SA	surface area	branch count	branch length	surface area	max length	max width	surface area
Female	branch count	0.412	0.850	0.683	0.786	0.902	0.903	0.710	0.907	0.426	0.345	0.361
	cell count	0.364	0.444	0.946	0.730	0.941	0.699	0.729	0.688	0.350	0.322	0.300
	branch length	0.304	0.437	0.481	0.742	0.901	0.594	0.611	0.573	0.283	0.304	0.280
	oogonia SA	0.302	0.292	0.309	0.360	0.904	0.900	0.628	0.914	0.477	0.471	0.507
	surface area	0.449	0.487	0.487	0.421	0.602	0.836	0.687	0.847	0.505	0.483	0.487
Male	branch count	0.480	0.386	0.341	0.447	0.537	0.687	0.733	0.986	0.295	0.230	0.276
	branch length	0.192	0.204	0.178	0.158	0.224	0.255	0.176	0.729	0.218	0.138	0.141
	surface area	0.485	0.382	0.331	0.456	0.547	0.680	0.255	0.693	0.442	0.376	0.421
Sporo.	max length	0.211	0.180	0.152	0.221	0.303	0.189	0.071	0.285	0.597	0.988	0.987
	max width	0.170	0.165	0.162	0.217	0.288	0.146	0.044	0.240	0.586	0.590	0.996
	surface area	0.167	0.145	0.141	0.221	0.274	0.166	0.043	0.254	0.553	0.554	0.526

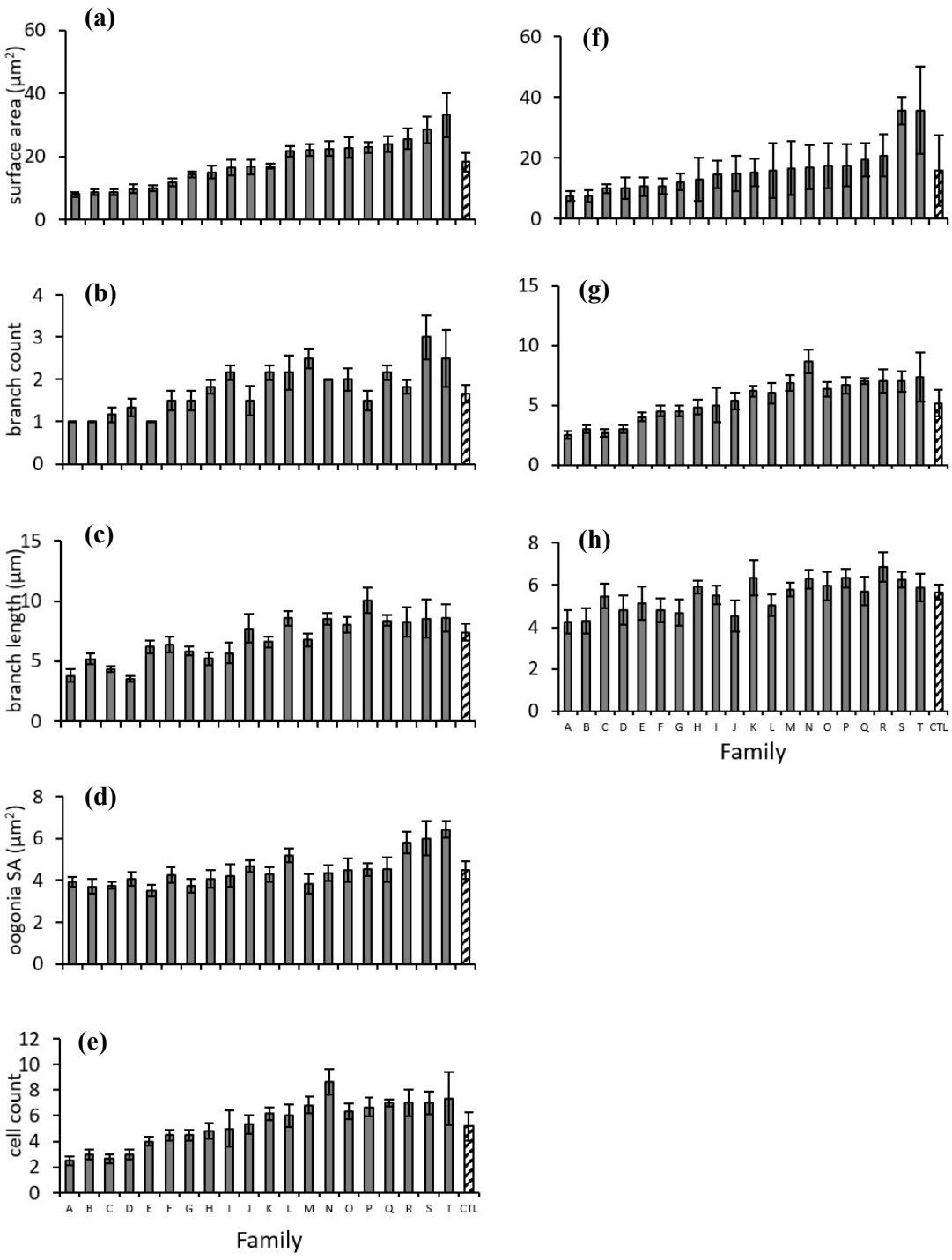

Figure 4.1 – Means (± SE) of five traits of female gametophytes (a – e) and three traits of male gametophytes (f – h) of *Ecklonia radiata* from 20 families (grey bars) plus a mix of all 20 families (thatched bars) after 14 days of growth. (a & f) total surface area, (b & g) branch count, (c & h) maximum branch length, (d) oogonia surface area, (e) cell count. Families are shown in increasing order of size based on total surface area of female or male gametophyte.

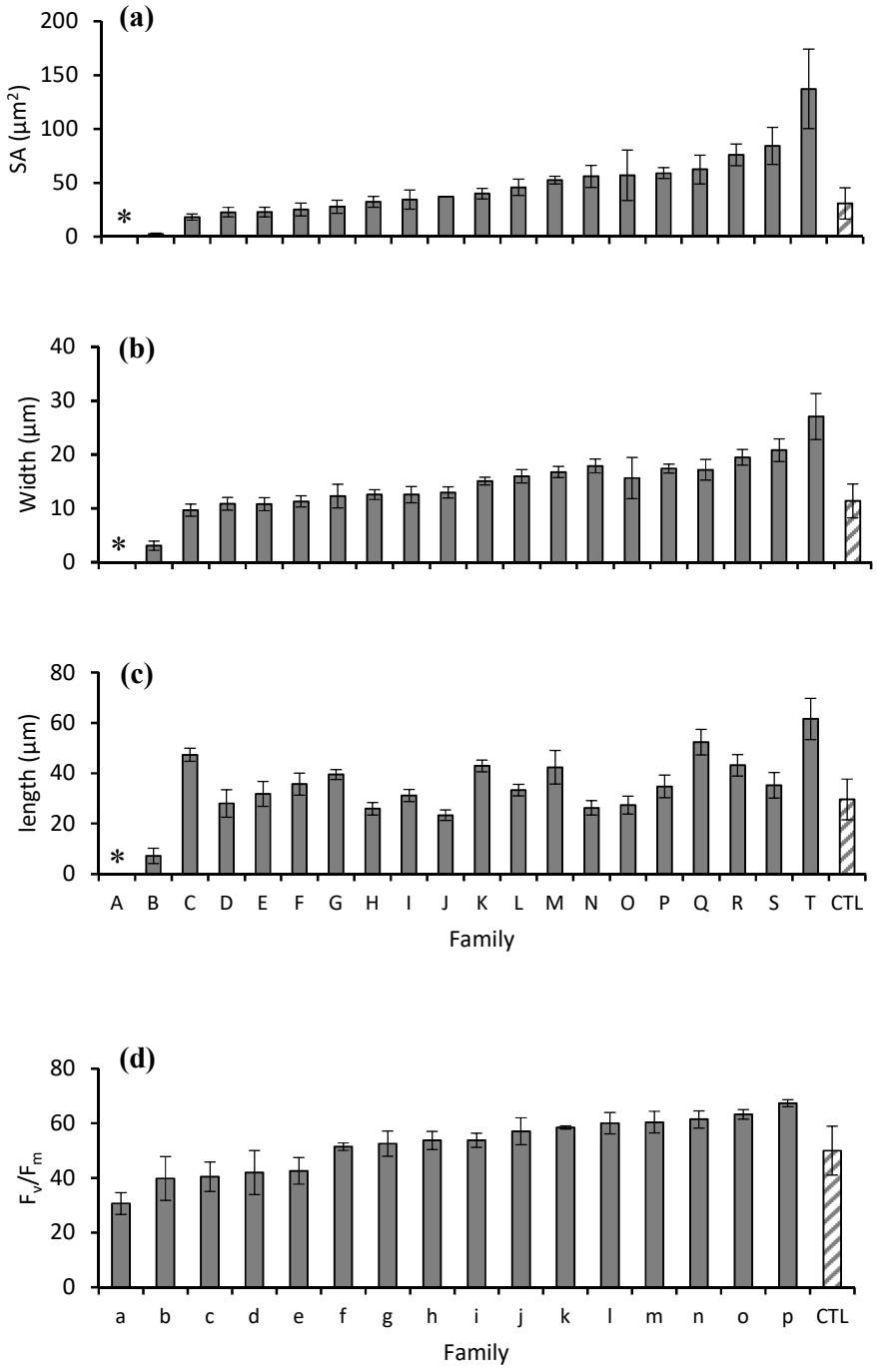

Figure 4.2 – Means (± SE) of four traits of sporophytes of *Ecklonia radiata* from multiple families (grey bars) plus a mix of all families (thatched bars). First experiment (a – c); families are shown in increasing order of surface area (SA) after 28 days of growth. (a) sporophyte SA; (b) sporophyte thallus width; (c) sporophyte length. Second experiment (d) families are shown in increasing order of maximum quantum yield (F_v/F_m) after 23 days of growth. * Indicates no emergence of sporophytes and were excluded from data analysis.

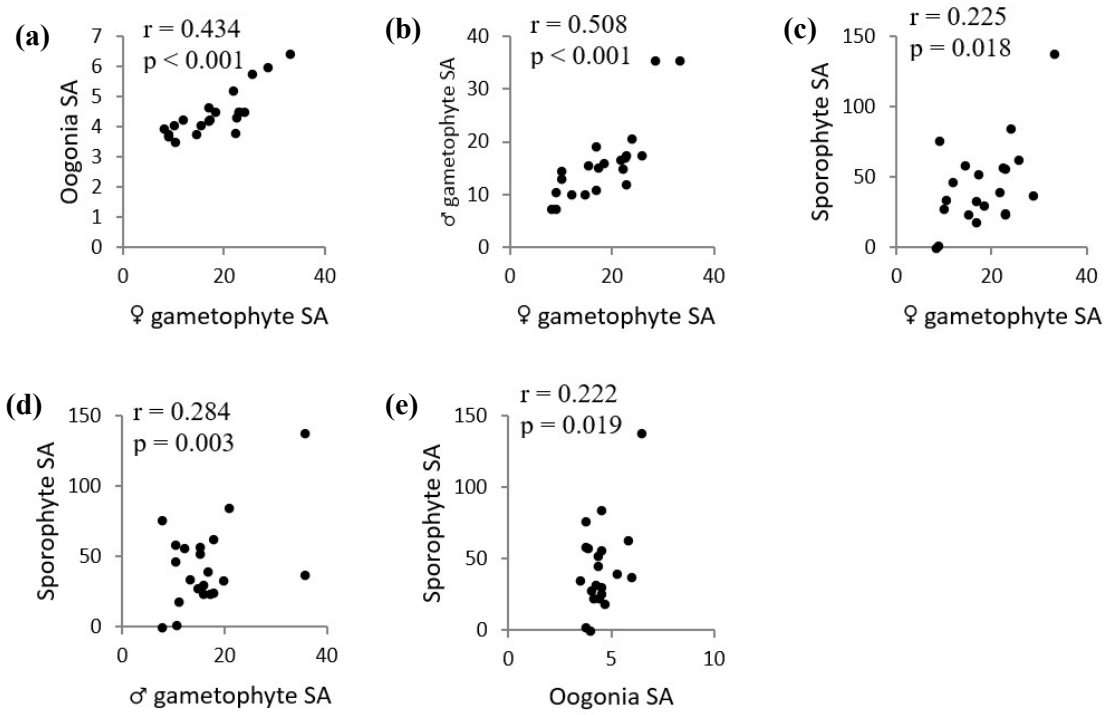

Figure 4.3 – Pairwise relationships between traits of male (♂) and female (♀) *Ecklonia radiata* gametophytes and sporophytes based on family means. All units of measurements are in µm. Pearson's *r* and significance are shown for each pair. (a) female gametophyte surface area (SA) v. oogonia SA; (b) female gametophyte SA v. male gametophyte SA; (c) female gametophyte surface area v. sporophyte SA; (d) male gametophyte SA v. sporophyte SA; and (e) oogonia SA v. sporophyte SA.

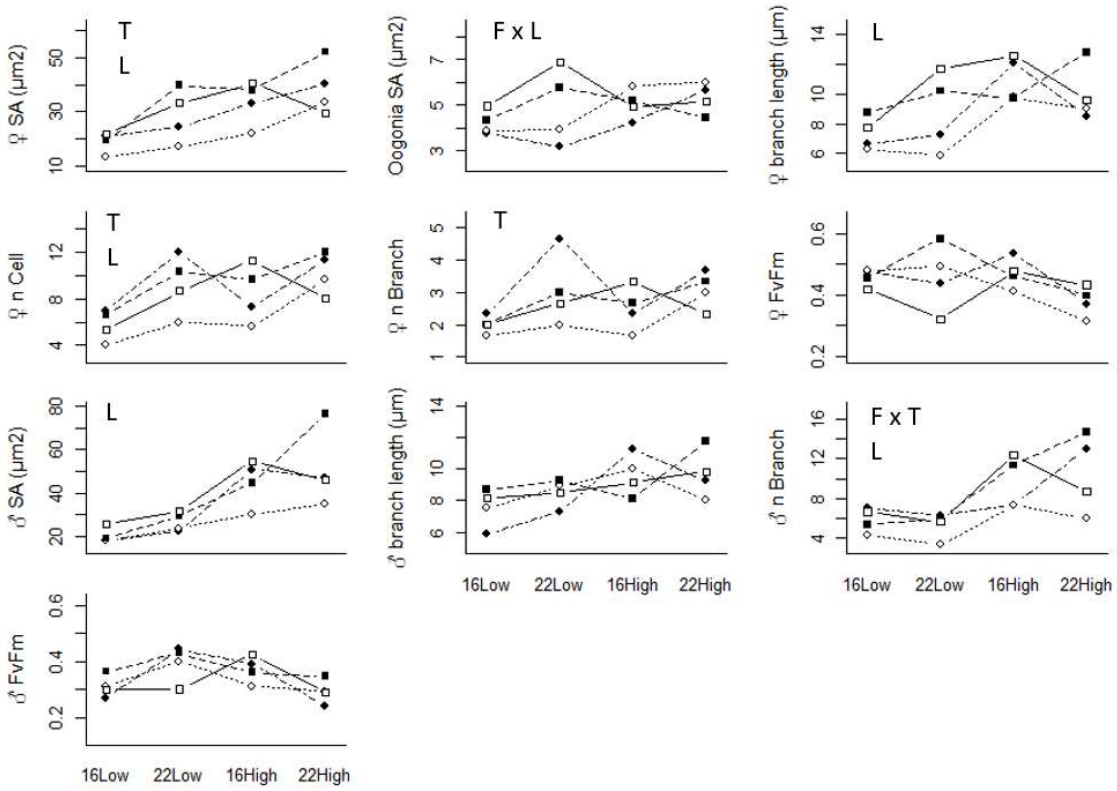

Figure 4.4 – Familial reaction norms for female (♀) and male (♂) *Ecklonia radiata* gametophyte traits: total surface area (SA); oogonia surface area; maximum branch length; cell count (n Cell); branch count (n branch) and; maximum quantum yield (F_v/F_m) across temperature (16 °C, 22 °C) and irradiance (Low, High) treatment combinations. Lines are drawn for visual purposes only and each line represents a different family. Letters denote which effects were significant ($p < 0.05$): Temperature – T; Light – L; Family x temperature – F x T; Family x light – F x L. Error bars are not shown for clarity.

4.5 Discussion

Strong family effects on key morphological and physiological traits were found across early life-cycle stages of *Ecklonia*. Trait values typically varied among families by 3-4 times and up to an order of magnitude for size-related traits of sporophytes. Additionally, significant family x environment interactions for some traits showed variable response to light and temperature among families.

4.5.1 Variation in key traits

Family-level variation for important functional traits in *Ecklonia* related to size, development, and physiological performance is in line with similar findings for terrestrial plants (leaf area, growth rates: Etterson 2004, Agrawal et al. 2008), corals (F_v/F_m: Császár et al. 2010), and intertidal seaweed (growth, F_v/F_m; Clark et al. 2013). These strong family-level effects occurred across development stages, suggesting a genetic basis for these traits and the potential for selection against deleterious alleles in the free-living haploid stage (Barner *et al.*, 2011). Despite this, there are likely to be non-genetic effects included in our estimates (i.e. maternal effects). For example, trait values in the gametophyte stages are likely influenced by the quality of zoospores and gametes which contain energy stores in the form of neutral lipid content originating from parental (maternal) generated sori (Brzezinski *et al.*, 1993), and the positive correlations between male and female gametophyte surface area supports this. Thus, the family-level variation in gametophyte traits and significant correlation between male and female gametophyte surface area may reflect both maternal and heritable genetic effects. However, as it was only possible to determine broad sense heritability these estimates include both additive and non-additive genetic effects in addition to possible maternal effects. Although broad sense heritability has its limitations, there are numerous studies of marine organisms for which sire and dam effects cannot be partitioned, or which are restricted by clonal or inbreeding designs, where it provides a basis for interpreting adaptation (Wright *et al.*, 2004a; Császár *et al.*, 2010; McKenzie *et al.*, 2012).

Furthermore, the first generation fitness costs of inbreeding in animals and plants are not apparent in kelps (Barner *et al.*, 2011), likely due to genetic recombination (meiosis) during spore production and purging of deleterious alleles in the haploid stage.

Although family-level variation in traits was determined from microscopic stages only, these traits may have important functional implications in response to climate change. Thallus size is generally a strong predictor of fitness, while larger egg or oogonia size increases offspring fitness in invertebrates (Huchette *et al.*, 2004) and bryophytes (Glime & Bisang, 2014). Larger oogonia also likely increases the probability of fertilisation success due to increased chances of antherozoids-oogonia encounters similar to egg size effects in broadcast spawning invertebrates (Levitan, 2006), or larger pheromone chemical volume released (Lüning & Müller, 1978). Maximum quantum yield (F_v/F_m), ostensibly a good indicator of PSII condition (Murchie & Lawson, 2013), also varied among families in sporophytes (F_v/F_m ranged between 0.132 and 0.714) but not gametophytes (F_v/F_m ranged between 0.004 and 0.716) suggesting adaptive potential for this trait. F_v/F_m values that reflect normal 'healthy' photosystem functioning in microscopic stages of kelp are not established, however F_v/F_m values in 'healthy' microalgae range from 0.21 – 0.65 (McMinn *et al.*, 2004, 2008). Similarly, photosynthesis profiles in corals exhibit broad-sense heritability, indicating potential for evolutionary adaptation to heat stress (Császár *et al.*, 2010), and regulation of genes that control for photosynthesis differ among genotypes in seagrass, influencing stress responses and recovery times to light stress (Salo *et al.*, 2015). Whether variation in F_v/F_m of *Ecklonia* sporophytes is directly associated with genetically determined pigment profiles or other attributes that influence photosystem activity in autotrophs is not known. Moreover, the transgenerational differences in family effects on F_v/F_m (no family effects for gametophytes but significant family effects for sporophytes) may reflect different selection pathways relating to the primary energy requirements of gametophytes (lipids) *vs*. sporophytes (light) for survival and production (Brzezinski *et al.*, 1993). However, the

relatively low number of families for the test of F_v/F_m in gametophytes (n =4) limits these conclusions. More broadly, our results emphasise the fundamental importance of exploring genetic responses to environmental change to enhance the understanding the response of species to climate change.

Seaweed traits often display high levels of phenotypic plasticity which allows a plastic response to spatial and seasonal variation in environmental factors (Fowler-Walker *et al.*, 2006). Highly plastic responses are typical of populations that have evolved in heterogeneous environments where strong directional selection can act on populations on small and large scales (Caswell, 1983; Reed *et al.*, 2010; Gratani, 2014). Relative to most other kelps, *Ecklonia* occurs across a large range of natural environmental gradients of temperature, wave exposure, depth, and light environments (Fowler-Walker *et al.*, 2006). The photosystem of juvenile brown algae possess rapid phenotypic buffering (plasticity) (Flukes et al. 2015; Mabin unpublished data) which suggests that environmental in addition to genetic factors influence F_v/F_m. It is not known how these results for the early life-cycle stages translate to macroscopic adults. However, in abalone and other shellfish, heritabilities of growth traits become stronger as the organism ages while phenotypic expression due to maternal and environmental effects are more pronounced in juveniles (Coman *et al.*, 2010; Brokordt *et al.*, 2015), highlighting the importance of estimating heritability through different developmental stages.

4.5.2 Family x environment interactions: temperature and light

Significant family x environment interactions occurred for oogonia surface area, where different families responded differentially to light, and male branch count, where families responded differentially to temperature. Both traits are related to reproduction; oogonia size likely influences both fertilisation success (Levitan, 2006) and post-fertilisation fitness of offspring (Levitan, 2006; Glime & Bisang, 2014), while greater branching in males presumably increases the abundance of antheridea and a higher production of antherozoids.

The phenotypic response of genotypes across environments (reaction norms) (Schmalhauzen, 1949) facilitates the exploitation of or offers protection from variable environments (Via & Lande, 1985). Under 'typical' environmental variability, reaction norm gradients of a population may be similar and genetic variation remains 'cryptic' (Ghalambor *et al.*, 2007). In contrast, divergence in the expression of phenotypic responses under stressful conditions (genotype x environment interaction) provides evidence of genotypic variation and an adaptive buffer under directional selection (Via & Lande, 1985; Hartman *et al.*, 2001), as some phenotypes will persist in the new environment while other deleterious phenotypes will be eliminated by selection (Ghalambor *et al.*, 2007). Understanding genotype x environmental interactions is a crucial step in characterising the links between stress, plasticity and adaptive evolutionary potential within populations.

Temperature and light showed strong effects on several traits in females but much weaker effects on males. Generally, females were larger with more branches and more cells at higher temperatures and higher light. Previously, it has been shown that the size (surface area) of *E. radiata* gametophytes did not differ when grown at temperatures between 16 and 22.5 °C (Mabin et al. 2013). In this study, although females were larger at higher temperature, no *E. radiata* sporophytes developed at 22 °C suggesting a break-down in fertilisation, post-fertilisation growth or delayed growth or development under marginal thermal conditions. Microscopic stages of kelp, especially gametophytes, can delay growth or development under low light or nutrients (Kinlan *et al.*, 2003; Carney & Edwards, 2006). A more extreme temperature than examined in this study (25.5 °C) resulted in arrested development of *E. radiata* sporophytes (Mabin et al. 2013). These differences in sporophyte development between studies suggests that the effects of temperature on development will change with factors including population of origin (Biscup et al 2014), genotype, and perhaps season (Mohring et al. 2014).

4.5.3 Non-genetic effects on traits

Non additive genetic effects such as dominance and interaction effects as well as maternal effects can also contribute to evolutionary processes, but are rarely considered for marine algae (Marshall, 2008) possibly due to the difficulty of partitioning sire (paternal) and dam (maternal) effects in monoecious species. Maternal effects have important influences on offspring traits in a range of organisms including trees (Larios & Venable, 2015) and marine invertebrates (Allen *et al.*, 2008) but their effects in seaweed are not known. Maternal effects can be caused by a combination of factors, including the parental environment (Rossiter, 1996), age of offspring (Marshall *et al.*, 2010), size or intraspecific competition (Allen *et al.*, 2008). Maternal effects have been shown to be most prominent under favourable or intermediate conditions of least stress and less prevalent under benign or extreme stress (see review Hoffmann & Merilä 1999). In our experiment cultures were grown under favourable conditions and maternal provisioning may have contributed in part to the strong family-level effects.

Phenotypic links between life-stages (transgenerational plasticity) can be driven by maternal effects, influencing the post-recruitment phenotype (Allen & Marshall, 2013). In marine invertebrates, maternal effects can determine juvenile fitness (Marshall, 2008) but maternal effects do not always continue across life history development with increasing offspring size, and are not always a good predictor of adult fitness (Allen & Marshall, 2014).

Overall, our data indicate that microscopic stages of *E. radiata* in Tasmania may be able to adapt to the changes in environmental conditions predicted to occur under climate change. Nonetheless, maternal effects appear to be present, especially in gametophytes, and are likely to constrain adaptive responses. Further insight into the adaptive potential of these traits could be achieved by partitioning of sire and dam effects to more fully understand and quantify the heritable and non-heritable components of trait variation. Dioecious species

without alternation of generations such as *Hormosira banksii* provide better opportunities for these kinds of tests in seaweed (Clark et al. 2014).

Chapter 5 | Population resilience of kelps to climate change – a synthesis

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5.1 Abstract

Kelp form the foundation of some of the most productive ecosystems on earth. They provide physical structure and critical resources in temperate reef systems, supporting high levels of biodiversity, endemism, and production. Environmental stresses influence kelp ecophysiological performance and distribution and these stresses are growing as a result of anthropogenic activity, threatening kelp populations in some regions. With effort focused on predicting how kelp ecosystems might look in the future under shifting stress regimes, this review describes some of the challenges in predicting the future condition of kelp populations given climate change, including the current limitations of seaweed research and the approach and interpretation of measuring 'performance' in photoautotrophs. I review the limitations of performance indicators such as relative growth rates, photosystem II characteristics (rETRmax, F_v/F_m) isotopic characteristics (C, N) and nucleic acid ratios (RNA:DNA) and indicators based on single measurements, and recommend against applying simplified or generalised models to determine future condition of kelp populations. For future research, I propose a coordinated and integrated approach that includes multifactor laboratory and field experiments and surveys that test climate impacts on multivariate physiology as well as the assessment of adaptive potential in kelp species across populations and life-cycle stages.

5.2 Kelps and response to climate change

Climate change is destabilising ecosystems globally (Walther *et al.*, 2002). Mounting evidence of large-scale population declines of ecosystem engineers including mangroves (Gilman *et al.*, 2008), coral reefs (Hoegh-Guldberg, 1999; Knowlton, 2001) and terrestrial forests (Allen *et al.*, 2010) and concomitant loss of biodiversity is associated with environmental shifts, often driven largely by climate change (Mooney *et al.*, 2009). Species can respond to selective pressures by phenotypic alteration and molecular change (evolution) which determines the adaptive potential of a species and its population. Seaweeds enhance biodiversity in temperate marine ecosystems by engineering habitat and providing resources (Bruno & Bertness, 2001; Krumhansl & Scheibling, 2012) and thus are important in maintaining ecosystem function. Large brown kelps (Laminarians) form extensive forests and their dynamics (growth, reproduction, and mortality) are regulated in part by abiotic factors and are susceptible to climate change.

Thermally driven decline in kelp has led to deforestation (Fernández, 2011; Johnson *et al.*, 2011; Wernberg *et al.*, 2012a) with ecosystem-wide implications (Scheffers *et al.*, 2016; Wernberg *et al.*, 2016a) via changes to resource availability for associated communities (Harley *et al.*, 2006) and reduced kelp population resilience via a break-down of facilitative environment-engineer feedbacks (Cuddington *et al.*, 2009). Large-scale warming or sustained heat waves can be exacerbated by additional disturbances related to climate change and other anthropogenic effects including nutrient starvation due to El Niño events (Dayton et al., 1999), increased kelp-herbivore interactions (Vergés *et al.*, 2016), disease outbreaks (Cole & Babcock, 1996; Cole & Syms, 1999), pollution (Falkenberg *et al.*, 2010), and overfishing (Ling *et al.*, 2009). Kelp distribution has contracted in 38% of marine ecoregions over the past half century (Krumhansl *et al.*, 2016), while in other areas there has been little change or even increases in kelp abundance. Kelp loss has been particularly severe in Australia where

the abundance of *Macrocystis pyrifera* forming dense surface canopies has declined by up to 95% over the last 60 years in Tasmania (Johnson *et al.*, 2011). Similarly, *Ecklonia radiata* forests have contracted poleward by ~100 km in Western Australia in response to a sustained and unprecedented, large-scale heatwave event in 2011 (Wernberg *et al.*, 2012a, 2016a), and there has been substantial loss of kelp in northern NSW as a result of overgrazing by subtropical rabbit fish that have moved southwards with warming waters (Vergés *et al.*, 2016). Synchronous changes in environmental factors can act synergistically to influence recruitment, growth, and survivorship. For example, increased temperature and CO_2 inhibits kelp recruitment directly and indirectly by negatively impacting reproduction (Roleda *et al.*, 2012) and favours the establishment of turfing algae over kelp recruits (Connell & Russell, 2010b). Up until recently there were very few studies addressing the effects of multiple climate change stressors on kelps and much of the work examined impacts of temperature (see review by Harley et al., 2006). Single factor approaches fail to capture synergistic or antagonistic effects of multiple stressors which can distort predictions (Hoffman *et al.*, 2003) for managers to anticipate realistic responses of kelp ecosystems to climate change (Lotze & Worm, 2002). The results from this thesis reaffirm the value of multifactor approaches to climate change research. Given the key role of kelps in temperate reef ecosystems and recent kelp decline in some regions, effective adaptive management of kelp bed systems require knowledge of how multifactor changes in climate and other stressors will further impact kelp performance.

5.3 Assessing kelp performance under multiple stressors

Global climate change impacts different levels of biological organisation (Scheffers *et al.*, 2016). Thus, ‘performance’ should be measured at the population level (e.g. recruitment, survivorship), organismal level (e.g. growth rate, competitive ability, reproductive output), and at the sub-organismal level (i.e. various measures of eco-physiological ‘competence’).

This deep understanding of kelp performance under changing environmental regimes from the sub-organismal to the population level is crucial in predicting how kelp ecosystems will respond to climate change and this thesis demonstrates the importance of a mechanistic and multivariate approach to assessing ecophysiological competence of kelp at the sub-organismal level.

Organismal-level response to environmental stress can be examined using laboratory, mesocosm, and field studies. Controlled laboratory and mesocosm experiments link environmental stressors with performance while field studies provide more 'ecologically relevant' correlations between performance and environment factors in natural settings (i.e. El Niño, upwelling, and heat waves: Dayton et al., 1999; Wernberg et al., 2016). Nonetheless, *in situ* trends can be difficult to interpret due to the difficulty in partitioning the effects of environmental factors (known and unknown). Thus, assessing kelp performance by integrating laboratory, mesocosm, and field experiments is likely to yield the most insightful research (e.g. Davison and Pearson, 1996; Graham, 1996; Littler and Littler, 1980).

'Performance' indicators are selected to characterise the overall competence of an organism's biological system. Seaweed systems comprise a complex array of interrelated biosynthetic processes, knowledge of which is incomplete (reviewed by Hurd et al., 2014c). Growth, productivity, and mortality are commonly used as measures of performance and are indicative of the net effects of environmental influence across a range of biosynthetic pathways. Given the complexity of seaweed biosynthetic pathways, these common performance metrics provide limited scope in determining which biosynthetic processes are being affected and contributing to the observed changes in 'performance'.

Technological advancements in compact and portable repetition-rate fluorometry has enabled cheap and non-destructive techniques for determining performance of the photosystem II

(PSII) in photoautotrophs. Maximum quantum yield (F_v/F_m), relative electron transport rate ($rETR_{max}$) and minimum saturating irradiance (E_k) provide important information about light-harvesting complex characteristics in PSII (Beer *et al.*, 2001; Belshe *et al.*, 2007). However, since these measurements are typically only taken from a small area of tissue they do not necessarily reflect stress at the level of the organism. In seaweed, thalli undergoing severe tissue necrosis can indicate a 'healthy' functioning photosystem (F_v/F_m) adjacent to extensive sections of necrotic tissue (see Chapters 2 & 3; Flukes et al., 2015).

Isotopic fractionation signatures in tissue determined from mass spectrometry enables determination of carbon and nitrogen use strategies (Hepburn *et al.*, 2011) which can change depending on prevailing temperature, light, or nutrient conditions. $\delta^{13}C$ signature values indicate the inorganic carbon source (Hepburn *et al.*, 2011; Raven & Beardall, 2014) with values less than -29 ‰ indicating diffuse uptake of CO_2 while values less negative than -25 ‰ indicate energy intensive carbon concentrating mechanism (CCM) uptake of HCO_3^- (Raven *et al.*, 2002). $\delta^{15}N$ signatures are used to trace environmental pathways of nutrients but little is known about this metric in macroalgae (Cohen & Fong, 2005). When combined with absolute tissue nitrogen values, $\delta^{15}N$ can be a useful indicator of patterns of nutrient utilisation (Chapter 2) and providing some differentiation among uptake, storage, or utilisation of ambient nitrogen (Gagné *et al.*, 1982; Kubler & Raven, 1995; Stephens & Hepburn, 2016).

For this thesis, quantification of DNA and RNA enabled evaluation of the Growth Rate Hypothesis (GRH). The GRH (Elser et al. 2000) posits that photosynthetic organisms at higher latitudes manifest greater peak growth rates than their lower latitude counterparts as a means of compensating for a shorter growing season. Moreover, assuming a constant DNA tissue concentration (as found for animals and microalgae: Dortch et al. 1983), it is expected that organisms with higher growth potential should have higher RNA:DNA ratios and higher

N-limitation thresholds (see Chapters 2 and 3). The RNA:DNA – organismal growth rate relationship is supported in invertebrates and microalgae (Dortch *et al.*, 1983; Lepp & Schmidt, 1998; Elser *et al.*, 2000a; Lovelock *et al.*, 2007) but evidence for a lack of support for this relationship in slow-growing seaweeds is mounting (Reef *et al.*, 2012; Flukes *et al.*, 2015) and I determined a lack of support for the GRH in both *M. pyrifera* and *E. radiata*. We found that RNA:DNA ratios can be affected either by a change in absolute concentrations of RNA or DNA and the assumption of constant DNA does not hold in *Ecklonia* and *Macrocystis*. Highly variable absolute RNA and DNA concentrations in these species were driven by multiple factor combinations of temperature, light, and nutrients (see Chapters 2 and 3). In addition, high RNA:DNA ratios were associated with an impaired photosystem (low F_v/F_m) in *M. pyrifera*, contrary to that found for brown Fucoid, *P. comosa* (Flukes *et al.*, 2015), and there were no associations of RNA:DNA with other metrics in *E. radiata*. In seaweeds, exposure to thermal or light stress can induce rapid increases in RNA concentration as heat shock proteins (HSPs) are produced (Lindquist, 1986; Vayda & Yuan, 1994) and the high variation in absolute RNA in *Ecklonia* could be an artefact of high genotypic variation in functional traits (Chapter 3 & 4). Thus, using metrics such as RNA:DNA and F_v/F_m in species exhibiting high family-level (genetic) variation such as *E. radiata* to determine ecophysiological competence are useful only when used in combination with other metrics, and supports that univariate measures fail to detect holistic response of organisms to stress (Roleda *et al.*, 2004a). Measuring multivariate responses of kelps to climate change is likely to offer a more comprehensive assessment whilst building knowledge of seaweed biology.

Emerging molecular techniques such as next generation sequencing (NGS) may provide effective indicators of stress response at the molecular level. NGS determines what genes are present (genomics) and what genes are upregulated (transcriptomics), enabling the

identification of differential gene expression and establishment of their functional annotations under selected stresses. Heat stress tolerance within and between populations can be derived as a function of stress, physiology, and differential gene expression to elucidate relative stress response (Mochida *et al.*, 2006). Recent studies have identified and quantified changes in gene expression of corals to temperature stress (Barshis *et al.*, 2013) and ocean acidification (Moya *et al.*, 2012). In seaweeds, reference genes have been identified from transcriptomic profiles (using RNASeq: Wang et al., 2009) of seaweeds subject to short-term stress trials (De Oliveira *et al.*, 2015; Alves-Lima *et al.*, 2016). Quantification of biosynthetic compounds and other important synthases relating to the expression of photosynthesis, carbohydrate synthesis and defence compounds (de Oliveira *et al.*, 2012; de Wit *et al.*, 2012) enables in-depth correlative analysis of organismal and population-level resistance to stressor impacts. NGS can also reveal differential response to stress for a species across latitudes (Gleason & Burton, 2015), highlighting the importance and utility of next generation approaches in further exploration of ecophysiological comparisons of stress along environmental gradients. Furthermore, datasets combining genetic, phylogenetic, and phenotypic responses to stress will enable identification of stress in an organism at a molecular level and provide resolution of possible evolutionary perspectives of stress and plasticity within and between populations. Thus downstream use of transcriptomics is now emerging as a viable and powerful approach to determining the effects of climate change on biota.

5.4 Functional plasticity and adaptability of kelps

Environmental stresses are an omnipresent structuring force in ecosystems (Suhett *et al.*, 2015) and act selectively on physiological traits (Hoffmann & Hercus, 2000), driving evolutionary processes and influencing population demography and distribution (Wahl *et al.*, 2011). Sessile species respond to environmental stress either by phenotypic adjustment (functional plasticity within lifetime) or adaptive response (between generations), with most

research on kelps focusing on phenotypic responses to novel stressors (Padilla & Savedo, 2013). Species that span a large latitudinal range and depth profile typically have high functional trait plasticity in order to persist in a range of different environments (Molina-Montenegro & Naya, 2012) and widely distributed seaweeds are renowned for having highly variable morphology (Ruuskanen & Bäck, 1999; Wernberg *et al.*, 2003). The adaptation potential for seaweeds is poorly known but recent studies reveal more adaptive potential in seaweeds than first thought (Chapter 4; Clark et al., 2013; Wright et al., 2004), with family-level variation in key functional traits demonstrating adaptive potential to climate change.

Population persistence under variable environments depends on the ability of individuals to adjust their phenotype to function under a range of conditions. Phenotypic adjustment in seaweeds is predominantly correlated with temperature, light, nutrients (Eggert, 2012; Flukes *et al.*, 2015; Shibneva & Skriptsova, 2015) and water flow (Fowler-Walker *et al.*, 2006) and enables individuals to acclimate to fluctuations in these key environmental factors. Tolerance to stress varies within and between kelp populations (Mohring *et al.*, 2014) and life stages (Chapter 4, Mabin et al., 2013; Roleda et al., 2004b; Tatsumi and Wright, 2016), and therefore to understand climate impacts on kelp species, performance must be assessed across these factors. Additionally, seaweed phenology is often seasonal and exhibits plasticity due to cumulative effects (e.g. stressful summers) (Wright *et al.*, 2004b), thus linking environmental cues to phenological response is essential to understand the timing of critical events at the population level (i.e. recruitment, gametogenesis, fertilisation) and how temporal and spatial shifts in stress regimes may impact these important processes. For instance, *E. radiata* sporophytes from a site failed to emerge under laboratory conditions at 22 °C (Chapter 4) from where they had emerged previously but during a different season (Mabin et al 2013). This may be attributable to seasonal variability of performance in microscopic stages (i.e. ‘ripeness of spores’). Conversely, different factors contributing to variation in performance

between NSW and Tasmanian *E. radiata* sporelings (Chapter 3) might be explained in-part by seasonal fitness of sporelings, with Tasmanian sporelings known to perform better after the winter months (Mabin pers. obs.) and highlights the importance of accounting for seasonal influences when designing experiments and interpreting kelp physiology data.

The morphology of adult *E. radiata* exhibits substantial regional differences between Tasmania and NSW (Mabin 2013; Flukes pers. comm.), while juvenile *E. radiata* and *P. comosa* exhibit differences in temperature and light tolerance between the two regions (i.e. ecotypic differentiation: Chapter 3; Flukes et al., 2015). There are conflicting data on whether morphological differences are genetically or environmentally driven in *Ecklonia* spp. (see Fowler-Walker et al., 2006 and Serisawa et al., 2002).

E. radiata and *M. pyrifera* juveniles rapidly altered their phenotype in response to abiotic stress, exhibiting functional plasticity, consistent with previous accounts of kelp morphology and the influence of wave stress (Fowler-Walker *et al.*, 2006; Wernberg & Vanderklift, 2010) and seasonal fluctuations in temperature and light (Miller *et al.*, 2011). Morphological traits converged in *E. radiata* transplanted from low to high wave stress environments over 7 months (Fowler-Walker *et al.*, 2006) although, no convergence occurred for transplants from stressful to benign environments in other *Ecklonia* spp. (although see Serisawa et al., 2002). Similarly, transplanting *E. radiata* juveniles from near their rear edge range (NSW) to mid-range (Tasmania) (Flukes in review) resulted in convergence of traits, with no differences in growth rates or ecophysiological traits (PSII parameters, isotopic signatures, and RNA:DNA ratios) evident after 23 days post transplant.

Differences in temperature tolerance between juvenile *E. radiata* from central-range and rear-edge cohorts in eastern Australia (Chapter 3) were consistent with field observations of the brown seaweeds *Scytothalia* (Andrews et al. 2014), *E. radiata* in western Australia (Staehr &

Wernberg, 2009), and laboratory and field studies of *Phyllospora* (Flukes). Rear-edge (warm-acclimated) ecotypes are capable of higher temperature tolerance than those from central range, however, rear-edge populations may be under stronger selection pressure and with reduced competency for reproduction and recruitment due to reduced density (Wernberg *et al.*, 2010) and genetic diversity. It has been suggested that the dominant mode of resistance of seaweed populations to climate change shifts from population-level adaptive capacity at high latitudes to thermal tolerance of individuals at low latitudes, as has been observed for *Scytothalia* in Western Australia (Bennett *et al.*, 2015). If the same is true for kelps, this would highlight the importance of assessing adaptive potential of widely distributed species across different populations. If rear-edge populations are less genetically diverse as found in southern-range *M. pyrifera* in California (Assis *et al.*, 2016), this may be a key factor for the observed decline of *M. pyrifera* on the East coast of Tasmania if their population-level adaptive capacity has been eroded and thermal conditions have surpassed tolerance levels.

Our results indicate that the current seasonal oceanic temperature, light, and nutrient profile in south east Australian waters are marginally within the dynamic functional plasticity thresholds for juvenile *E. radiata* but not *M. pyrifera*. The phenotypic response of *M. pyrifera* to heat and light was a lot more pronounced (Chapter 2) than Tasmanian *E. radiata* juveniles (Chapter 3), and experienced a much greater degree of fitness degradation and mortality under simulated future temperatures and light extremes.

Much focus of climate change research draws ecological interpretations from observations of physiological performance of individuals under a range of stresses. However, there is less information linking stress, performance, and adaptive capacity. Empirical evidence of family-level variation in functional traits of kelp in two out of three life-stages (Chapter 4) suggests adaptation may be a crucial component determining resilience of kelp populations to climate change. Differences in the response across life-stages also indicates the need to examine both

functional plasticity and adaptive capacity in the context of future climate across latitudes, seasonal extremes, and life stages. As selection pressure has variable impacts on quantitative traits across life-stages (Harvey *et al.*, 2014) and phenotypic links between life stages are complex and context dependent (Marshall 2008; Allen & Marshall 2013; Nylin and Gotthard 1998), heritability of functional traits will culminate in variable tolerance to different stressors across life-stages. For example, early microscopic life-cycle stages of *E. radiata* are negatively impacted by elevated temperatures but not reduced nitrate concentration (Mabin *et al.*, 2013; Mohring *et al.*, 2014), while effects of high light depend on the life-cycle stage (Tatsumi & Wright, 2016). Additionally, high temperature and high light have additive negative effects that limit recruitment of *Macrocystis* and *Ecklonia* due to photoinhibition (Mabin pers. obs.; Graham, 1996) thus assessment of vulnerabilities to multifactor stress regimes is required across life stages.

This thesis shows strong family-level variation in quantitative traits which could suggest adaptive potential in those traits. This kind of adaptive potential is a universally important determinant of population-level resistance to climate change (and other stressors) and the response of quantitative traits to environmental stress is demonstrated across a wide range of taxa, including birds (Pulido et al. 2001), trees (Hawkins *et al.*, 2010), *Drosophila* (Coyne & Beecham, 1987), and marine invertebrates (Galletly *et al.*, 2007; Pease *et al.*, 2010; McKenzie *et al.*, 2012). Mounting evidence indicates that the rate of recent climate change and associated abiotic changes in the marine environment (e.g. anomalies brought on by extreme El Nino (Dayton et al 1999), and heatwave events (Wernberg et al. 2013)), is resulting in extensive die-offs and range contractions in some regions like that observed for *M. pyrifera* in Tasmania (Johnson *et al.*, 2011). For some species, it is likely that changing stress regimes are outpacing the population's capacity to adapt (Parmesan, 2006), thus

highlighting the importance of adaptive capacity in the context of species response to climate change.

5.5 Conclusions

Climate change is shifting environmental stress regimes in the world's oceans, and in south east Australia ongoing change in ocean circulation patterns (Oliver *et al.*, 2014) are anticipated to result in further increases in temperature and reductions in nitrates. Furthermore, it is predicted that local benthic light regimes will change due to canopy destabilisation and continue to impact important biogenic habitat-forming kelps. To understand the response of kelp populations to climate change we need to understand their physiological response, limits to functional plasticity, and adaptive capacity under future environmental regimes.

This thesis goes some way to elucidating the potential effects of future climate on two important kelp species, *E. radiata* and *M. pyrifera*, in south eastern Australia at key stages of their development. Overall, high temperature drives physiological degradation at the individual-level, which is mediated by light and nutrient regimes. While kelp at lower latitudes may tolerate higher temperatures, the high family-level variation in response to stress may provide an adaptive buffer, allowing populations to respond in an evolutionary way to climate change. The inclusion of seasonal and life-cycle perspectives on kelp fitness, examining sire and dam effects to determine narrow-sense heritabilities in key traits and next generation sequencing are alternative approaches that would provide additional data to inform the future of kelps and kelp ecosystems under climate change. Furthermore, a detailed perspective on the 'stress ecology' of kelps is essential to develop better models to predict climate change impacts on temperate marine ecosystems. As discussed in this thesis, we need to increase knowledge of kelp performance with respect to ecophysiology, adaptive potential,

seasonality and life-stage by linking, molecular, organismal and ecosystems stress responses to predict future performance and species distribution. This will provide a better indication of the location-specific performance of a population under climate change. This kind of integrated approach can be used to develop a more holistic understanding of kelp biology and ecophysiology in response to climate change, allowing for better predictions of future condition of temperate rocky-reef marine ecosystems.

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